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PRINCIPAL INVESTIGATOR: Yosef Yarden, Ph.D.

CONTRACTING ORGANIZATION: The Weizmann Institute of Science
76100 Rehovot, Israel

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6. AUTHOR(S)
Yosef Yarden, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Weizmann Institute of Science
76100 Rehovot, Israel

E-Mail: yosef.yarden@weizmann.ac.il

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13. ABSTRACT (Maximum 200 Words)

Signals to multiply, migrate and outgrow blood vessels are mediated by growth factors of the EGF/neuregulin family. Concentrating on the EGF-receptor, our first task was to resolve mechanisms that normally restrain signalin. These efforts have led to the realization that activated growth factor receptors are modified by mono-ubiquitins rather than by poly-ubiquitylation. Receptor ubiquitylation is mediated by the c-Cbl E3 ligase, and we identified a Cbl-binding protein, called Grb2, as an enhancer of Cbl's action on active receptors. Our Task 2 relates to a putative particle that modulates recycling of ErbBs, and we identified c-Src as a major player in recycling: c-Src phopshorylates c-Cbl and leads to its proteasomal destruction. Consequently, tumor cells overexpressing c-Src are unable to down-regulate ErbB proteins. In addition, we identified the Hgs adaptor and the Nedd4 ubiquitin ligase as regulators of receptor recycling. In an effort to translate our findings to clinical protocols, our Task 3 involved in vitro studies and assays in animals testing combinations of monoclonal antibodies to ErbBs, inhibitors of their intrinsic tyrosine kinase activity, and blockers of c-Src. Unlike the results of the first two tasks, which have been published, the results of the latter part are currently assembled for publication.

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Table of Contents

INTRODUCTION

Growth factors and their receptors play an essential role in fixation and clonal expansion of oncogenic mutations. Consistent with their pivotal role in cancer development and metastasis, inhibition of signaling by growth factors of the EGF/neuregulin family leads to tumor arrest in model systems. Moreover, monoclonal antibodies directed at the receptors and low molecular weight inhibitors of the associated tyrosine kinase activity are already used to treat cancers of breast and lung, respectively. Our original Statement of Work addressed the mechanisms underlying negative regulation of activated growth factor receptors in cancer cells. This understanding will likely be translated into effective strategies to inhibit tumors whose growth and metastasis depend on growth factors. Our work concentrated on one growth factor receptor, EGFR (also called ErbB-1 and HER1), and its family members, including Her2/ErbB-2. Our major objective was the understanding of the process that sorts endocytosed receptors to degradation or to recycling back to the cell surface. The major regulator is c-Cbl, an E3 ubiquitin ligase, which binds many signaling proteins. In the lsat year of research we learned that c-Cbl mediates conjugation of monomeric ubiquitins to multiple sites on the ligand-activated receptor. Our search for c-Cbl-interacting regulators (Task 1 of the original Statement of Work) led us to the identification of Grb2 as an effective collaborator of c-Cbl that augments receptor ubiquitylation and degradation. Concentrating on a multi-protein complex that regulates the alternative pathway, which leads to receptor recycling (Task 2), we identified c-Src as a major regulator: this cytoplasmic kinase binds with c-Cbl, phosphorylates it and enhances its degradation by the 26S proteasome. Other key components of the sorting machinery we identified are Hrs/Hgs, an adaptor that binds ubiquitin, and Nedd4, an E3 ligase of Hrs/Hgs. Although yet unclear, these results shed light on the sorting machinery and its major components. To evaluate the therapeutic implications of the sorting machinery, we initiated over the last year experiments in pre-clinical models. The major aim is to examine the ability of Src inhibitors to augment the inhibitory action of anti-receptor therapies. Likewise, we currently analyze the relevance of c-Cbl and its binders to antibody-mediated therapy of human cancer.

BODY

Specific Aims **(as stated in the original grant application)**

(i) To isolate and molecularly clone the hypothetical phosphotyrosine-binding sorting protein by using its identified binding site on Cbl. The functional relationships to ErbB proteins and the recycling machinery will be studied in details.

(ii) To characterize a putative multi-protein complex, the recyclosome, and its individual components, including PI3K, Cbl, and Src, as well as 14-3-3 and small GTP-binding proteins. The functional relationships within the recyclosome and their relative order of action will be worked out.

(iii) To evaluate the therapeutic potential of inhibitors of specific components of the recyclosome. The ability of tumor-inhibitory anti-ErbB-2 antibodies to interfere with recycling will be examined in an attempt to improve existing immunotherapy or identify efficient combinations of antibodies and chemotherapeutic drugs.

Task 1: *To isolate and molecularly clone a hypothetical phosphotyrosine- binding protein responsible for degradation of ErbB proteins (see Appendix and H. Waterman et al., 2002).*

The ubiquitin ligase c-Cbl targets epidermal growth factor receptors (EGFRs) to endocytosis by tagging them with multiple ubiquitin molecules. However, the type of ubiquitylation is unknown; whereas poly-ubiquitin chains signal proteasomal degradation, ubiquitin monomers control other processes. We found that in isolation c-Cbl mediates mono- rather than poly-ubiquitylation of EGFRs. Consistent with the sufficiency of mono-ubiquitylation, when fused to the tail of EGFR, a single ubiquitin induces receptor endocytosis and degradation in cells. By using receptor and ubiquitin mutants, we concluded that c-Cbl attaches a founder mono-ubiquitin to the kinase domain of EGFR, and this is complemented by the conjugation of additional mono-ubiquitins. Hence, receptor tyrosine kinases are desensitised through conjugation of multiple mono-ubiquitins, which is distinct from poly-ubiquitin-dependent proteasomal degradation. In order to identify Cbl-associated proteins that regulate receptor degradation, we concentrated on a mutant of EGFR which cannot recruit c-Cbl. Unexpectedly, the mutant receptor displayed significant residual ligand-induced ubiquitination, especially in the presence of an overexpressed c-Cbl. The underlying mechanism seems to involve recruitment of a Grb2-Cbl complex to Grb2-specific docking sites of EGFR and concurrent acceleration of receptor ubiquitination and desensitization. Thus, in addition to its well-characterized role in mediating positive signals, Grb2 can terminate signal transduction by accelerating c-Cbl-dependent sorting of active tyrosine kinases to destruction.

Task 2: *To characterize a putative multi-protein complex, the recyclosome, and its individual components (see publications by J. Bao et al., 2003, and M. Katz et al., 2002).*

Our Task 2 aims at resolving the mechanisms underlying the action of a putative multi-protein complex that controls endocytosis of growth factor receptors. Ligand-dependent endocytosis of the epidermal growth factor receptor (EGFR) involves recruitment of an ubiquitin ligase, and sorting of ubiquitylated receptors to lysosomal degradation. By studying Hgs, a mammalian homologue of a yeast vacuolar-sorting adaptor, we provided information on the less understood, ligand-independent pathway of receptor endocytosis. Constitutive endocytosis involves receptor ubiquitylation and translocation to Hgs-containing endosomes. Whereas the lipid-binding motif of Hgs is necessary for receptor endocytosis, the ubiquitin-interacting motif (UIM) negatively regulates receptor degradation. We demonstrated that the UIM is endowed with two functions: it binds ubiquitylated proteins and it targets self-ubiquitylation by recruiting Nedd4, an ubiquitin ligase previously implicated in endocytosis. Based upon the dual function of the UIM and its wide occurrence in endocytic adaptors, we propose a UIM network that relays ubiquitylated membrane receptors to lysosomal degradation through successive budding events. A second line of research concentrated on the observation that cellular Src (c-Src) and epidermal growth factor receptor (EGFR) collaborate in the progression of certain human malignancies. To address the underlying mechanism, c-Src proteins were ectopically expressed together with EGFR in fibroblasts devoid of ubiquitous Src family proteins. We found that EGFR accumulates in cells overexpressing either wild-type c-Src or its transforming mutant. Up-regulation of EGFR requires its kinase function, as well as an auto-phosphorylation site known to bind c-Cbl, a major negative regulator of active receptors. Apparently, c-Src phosphorylates c-Cbl, and consequently accelerates its proteasomal destruction. Although ubiquitylation of c-Cbl is enhanced by c-Src, the intrinsic ubiquitin ligase function of c-Cbl may not be necessary for Src-induced degradation. Hence, by promoting destruction of c-Cbl, c-Src recycles growth factor receptors and enhances their mitogenic signals, which may explain Src s synergy with EGFR in oncogenesis.

Task 3: *To evaluate the therapeutic potential of inhibitors of specific components of the recyclosome.*
Detailed understanding of receptor desensitization mechanisms will likely identify potential targets for drug intervention. Our working hypothesis assumes that antibody-induced endocytosis contributes to tumor inhibition by immunotherapeutic drugs. Likewise, ligand-induced endocytosis of ErbB proteins underlies cytotoxicity of drug-neuregulin conjugates. Hence, efforts to enhance receptor endocytosis or inhibit recycling will likely improve drug efficacy. Along this line, we initiated two lines of research by using pre-clinical models in vitro and in tumor-bearing animals:

(i) Testing combinations of tyrosine kinase inhibitors directed against ErbB proteins (CI-1033, a pan-ErbB inhibitor made available by Pfizer), and Src-family members (PP2). The working hypothesis assumes that CI-1033 will block signaling, while PP2 will block an escape pathway that allows receptors to continue signaling by recycling back to thye cell surface.

(ii) Testing the relevance of c-Cbl and the characterized recyclosome to antibody-mediated inhibition of ErbB-driven tumors. The working hypothyesis assumes that tumor-inhibitory antibodies enhance endocytosis and degradation of their target receptors. Hence, we tested receptor ubiquitylation and effects of Src and Nedd4 on antibody-mediated receptor degradation and inhibition of cell growth.

Although these studies have not reached their publication phase, we have already learned that combining Src- and ErbB-specific inhibitors results in an additive growth inhibitory effect. In addition, our results raise the possibility that the mechanism of antibody-induced down regulation of growth factor receptors is distinct from the ligand-induced pathway. The regulators of the antibody-induced pathway may not be c-Cbl and Nedd4; the identity of the unknown mechanism will be addressed by our future studies.

KEY RESEARCH ACCOMPLISHMENTS

♦ Elucidation of the mechanism underlying receptor ubiquitylation and degradation: multiple monomeric ubiquitins, rather than chains of ubiquitin, are attached to each receptor molecule and lead to its degradation.

♦Identification of Grb2 as a Cbl-binding adaptor that enhances receptor endocytosis and degradation.

♦Identification of a molecular mechanism underlying oncogene collaboration in human cancer cells. This regulatory loop involves c-Src and c-Cbl, adaptors that antagonistically regulate ErbB trafficking.

♦ Uncovering the mode of action of the endocytic adaptor Hgs, including regulation by an ubiquitin ligase (Nedd4) and a network of adaptors containing an ubiquitin interacting motif (UIM; e.g., STAM).

♦ Initial characterization of the function of a lipid-binding domain (the FYVE) in receptor endocytosis. Our results indicate that PI3P, a product of inositol lipid kinases, is essential for effective endocytosis of ErbB proteins.

♦ Definition of potentially synergistic pairs of drugs, including Src and ErbB inhibitors (low molecular weight kinase blockers and monoclonal antibodies).

REPORTABLE OUTCOMES:

Mosesson, Y., Shtiegman, K., Katz, M., Zwang, Y., Vereb, G., Szollosi, J., and Yarden, Y. (2003). Endocytsosis of receptor tyrosine kinases is driven by mono-, not poly-ubiquitylation (in press; see Appendix).

Bao, J., Gur, G., and Yarden, Y. (2003). Src promotes destruction of c-Cbl: Implications for oncogenic synergy between Src and growth factor receptors, Proc Natl Acad Sci U S A *100*, 2438-43.

Katz, M., Shtiegman, K., Tal-Or, P., Yakir, L., Mosesson, Y., Harari, D., Machluf, Y., Asao, H., Jovin, T., Sugamura, K., and Yarden, Y. (2002). Ligand-Independent Degradation of Epidermal Growth Factor Receptor Involves Receptor Ubiquitylation and Hgs, an Adaptor Whose Ubiquitin-Interacting Motif Targets Ubiquitylation by Nedd4, Traffic *3*, 740-751.

Waterman, H., Katz, M., Rubin, C., Shtiegman, K., Lavi, S., Elson, A., Jovin, T., and Yarden, Y. (2002). A mutant EGF-receptor defective in ubiquitylation and endocytosis unveils a role for Grb2 in negative signaling, Embo J *21*, 303-13.

Reviews

Waterman, H., and Yarden, Y. (2001). Molecular mechanisms underlying endocytosis and sorting of ErbB receptor tyrosine kinases, FEBS Lett *490*, 142-52.

Shtiegman, K., and Yarden, Y. (2003). The role of ubiquitylation in signaling by growth factors: implications to cancer, Semin Cancer Biol *13*, 29-40.

Citri, A., Skaria, K. B., and Yarden, Y. (2003). The deaf and the dumb: the biology of ErbB-2 and ErbB-3, Exp Cell Res *284*, 54-65.

Harari, D., and Yarden, Y. (2000). Molecular mechanisms underlying ErbB2/HER2 action in breast cancer, Oncogene *19*, 6102-14.

CONCLUSIONS

Comprehensive understanding of mechanisms underlying degradation of growth factor receptors will likely lead to new prognostic strategies and novel therapeutic approaches to breast cancers. Our results portray a complex machinery that regulates growth factor receptors. The ligand-mediated pathway of receptor degradation is regulated by c-Cbl, an E3 ubiquitin ligase that recruits to tyrosine 1045 of EGFR and appends multiple monomeric ubiquitins to the phosphorylated receptor molecule. An alternative way to recruit c-Cbl emerged from our work. This involves binding of a Grb2-Cbl complex to phosphorylated tyrosine residues other than tyrosine 1045. The recycling mechanism of EGFR opposes the Cbl-mediated degradative process and it involves the action of c-Src. Src is physically associated with c-Cbl and leads to its poly-ubiquitylation. Consequently, Cbl is directed to degradation in the proteasome. The ligand-independent pathway of receptor degradation involves another E3 ubiquitin ligase, called Nedd4, and its adaptors, namely UIM-bearing proteins like Hgs and STAM. This alternative route of receptor endocytosis is slower and occurs even in the absence of growth factors. Finally, the emerging models of receptor degradation were tested by using a pharmacological approach. Antagonists of c-Src and of receptor phosphorylation were assayed in vitro and in tumor-bearing animals. Our studies suggest that Src inhibitors augment the action of anti-ErbB antibodies, while PI3K inhibitors may inhibit endocytosis, and therefore they restrict the action of endocytosis-based drugs (e.g., toxin conjugates). In addition, we initiated analyses of the mechanisms underlying antibody-induced degaradtion of EGFR and its homolog, ErbB-2. The results we collected so far suggest that a third mechanism regulates this pathway, and unlike the other pathways, ubiquitylation may not be involved in the antibody-induced mechanism of receptor down regulation. The results of these latter studies will soon be summarized for a publication, and we expect them to constitute a basis in a future grant application.

Appendix:
Endocytosis of receptor tyrosine kinases is driven by mono-, not poly-ubiquitylation
(Y. Mosseson et al., manuscript in press).

Abstract

Growth factors stimulate specific receptor tyrosine kinases, but subsequent receptor endocytosis terminates signalling. The ubiquitin ligase c-Cbl targets epidermal growth factor receptors (EGFRs) to endocytosis by tagging them with multiple ubiquitin molecules. However, the type of ubiquitylation is unknown; whereas poly-ubiquitin chains signal proteasomal degradation, ubiquitin monomers control other processes. We report that in isolation c-Cbl mediates mono- rather than poly-ubiquitylation of EGFRs. Consistent with the sufficiency of mono-ubiquitylation, when fused to the tail of EGFR, a single ubiquitin induces receptor endocytosis and degradation in cells. By using receptor and ubiquitin mutants, we infer that c-Cbl attaches a founder mono-ubiquitin to the kinase domain of EGFR, and this is complemented by the conjugation of additional mono-ubiquitins. Hence, receptor tyrosine kinases are desensitised through conjugation of multiple mono-ubiquitins, which is distinct from poly-ubiquitin-dependent proteasomal degradation.

Introduction

Protein ubiquitylation has emerged as a versatile regulatory strategy [reviewed in (1)]. In its best-characterised role as a signal for proteasomal degradation, productive recognition of ubiquitylated substrates is shown to minimally require a tetra-ubiquitin chain (2). Alternatively, studies in yeast attribute to mono-ubiquitylation an intrinsic capacity to target substrates both for internalisation at the plasma membrane and sorting at multi-vesicular bodies towards destruction in the vacuole [reviewed in (3)]. With subsequent identification of ubiquitin-binding activities, such as the UIM, a rationale for ubiquitin-dependent recognition of substrates has materialised (4-7). In higher eukaryotic systems, ubiquitylation of cell-surface receptors, likewise, correlates with their down-regulation via orthologous trafficking pathways that employ counterparts conserved from yeast (8-11). Ligand-activated ubiquitylation of EGFR, as well as other RTKs, is mediated by c-Cbl (12-14). Whether or not EGFR ubiquitylation is sufficient for its internalisation remains an open question. Likewise, although it is clear that each endocytosed receptor is conjugated to several molecules of ubiquitin, it is currently unknown to which extent branching of the EGFR-conjugated ubiquitins occurs in living cells. Here we present evidence indicating that the action of c-Cbl is limited to the addition of monomeric ubiquitins, and these are sufficient for receptor endocytosis and degradation.

Experimental Procedures

Reagents and antibodies

Unless indicated, reagents were purchased from Sigma (St. Louis, MO). E1 was from Affiniti (Mamhead, Exeter, UK), and rabbit reticulocyte lysate from Promega (Madison, WI). The 528-IgG

antibody was isolated from hybridomas and a Fab fragment prepared and labelled with Cy3. An antibody to EGFR was from Alexis (San Diego, CA). Anti-EEA1 mouse antibody was from Transduction Laboratories (Lexington, KY). Fluorescently-labelled antibodies were purchased from Jackson ImmunoResearch (West Grove, PA).

Construction of expression vectors

A plasmid encoding a truncated EGFR (EGFR-ΔC) was prepared by introducing a STOP codon after amino acid 1087. The EGFR-ΔC4R mutant was generated by site-directed mutagenesis. Vectors expressing EGFR::Ub chimeras were prepared by overlap extension PCR and mutations introduced. HA-tagged ubiquitin, either WT or K0, was sub-cloned into pEFIRES. Bacterial expression vectors for wild type $(His)_6$HA-Ub and $(His)_6$HA-Ub-K0 were obtained by sub-cloning into the pET28 plasmid (Novagen). Recombinant ubiquitins were subsequently affinity-purified on Ni^{2+}-conjugated agarose beads.

Transient transfection, immunoprecipitation and immunoblotting

Transfections were carried out using 1 μg DNA of each expression vector, and the total amount of DNA normalised with the respective empty plasmid. Cells were assayed forty-eight hours after transfection. Whole cell lysates were analysed by SDS-PAGE, either directly or after IP, and protein bands detected with an enhanced chemiluminescence reagent.

In vitro ubiquitylation assay

Receptor immunoprecipitates were extensively washed and re-suspended in ubiquitylation buffer [40 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$ and 2 mM DTT] supplemented with 2 mM ATP, and containing either rabbit reticulocyte lysate (1 μl), or recombinant E1 (0.1 μg) and E2 (UbcH5C; 7 μl of crude bacterial extract). Wild type or mutant $(His)_6$HA-tagged ubiquitin (0.5 μg) and GST-Cbl (0.1 μg) were added as indicated. Reaction mixtures were incubated for 1 hour at 30°C.

Immunofluorescence

Cy3-labelled Fab (20 μg/ml) was incubated with cells at 4°C for 90 minutes. Thereafter, cells were transferred to 37°C for the indicated time intervals before fixation and visualisation. Alternatively, paraformaldehyde-fixed cells were permeabilised for 10 min at 22°C with PBS containing 1% albumin and 0.2% Triton X-100. For labelling, cells were incubated for 1 hour with an anti-EGFR antibody, and after extensive washes, incubated for an additional hour with a Cy3-conjugated donkey anti-mouse $F(ab)_2$. Alternatively, cover slips were co-incubated with monoclonal anti-HA and anti-EEA1 antibodies, washed thoroughly, and incubated with a Cy3-conjugated donkey anti-rat $F(ab)_2$ and a Cy2-conjugated donkey anti-mouse $F(ab)_2$.

Results

In cells c-Cbl promotes conjugation of several ubiquitins to each receptor molecule, but in isolation it appends monomeric ubiquitins to EGFRs—In line with previous reports, when co-expressed with EGFR in CHO cells, c-Cbl destabilised the receptor and enhanced receptor ubiquitylation (Fig. 1A). When immunoblotted, the destabilised EGF-activated receptors exhibited a ladder of bands representing molecules differing in the number of conjugated ubiquitins. This stoichiometric multiplicity can be attributed to conjugation of either a ubiquitin polymer (henceforth: poly-ubiquitylation), many mononeric ubiquitins attached to several receptor's lysines (henceforth: multi-ubiquitylation), or a combination of mono- and poly-ubiquitins.

Polymerisation of ubiquitin on a substrate utilises certain lysines as branching sites [reviewed in (1)]. Therefore, to determine whether c-Cbl possesses mono- or poly-ubiquitylating activity, we compared the in vitro modifying capacity of wild-type ubiquitin (WT-Ub) with that of Ub-K0 that, by definition, is unable to form polymeric chains. Both forms were expressed in bacteria, and included as the sole source of ubiquitin in reconstituted reactions containing an isolated EGFR. Surprisingly, upon co-incubation with a bacterially expressed c-Cbl, both WT-Ub and Ub-K0 reproduced similar patterns that, according to the observed molecular shift, corresponded primarily to a mono-ubiquitylated EGFR (Fig. 1B). Hence, the results suggest that c-Cbl is equipped with an intrinsic mono-, rather than poly-ubiquitylating activity.

An internalisation-defective mutant of EGFR acquires rapid endocytosis and degradation when fused to a single ubiquitin—We predicted that once EGFR is tagged by a single molecule of ubiquitin, it will be sorted for endocytosis even when coupling to c-Cbl is not permitted. To test this prediction we utilised an internalisation-defective mutant of EGFR, incapable of direct c-Cbl binding [EGFR-Y1045F; (15)]. A single copy of ubiquitin was fused to the carboxyl-terminus of Y1045F-EGFR, but in order to prevent formation of covalent adducts, we replaced the terminal glycine (Ub-G76), or both glycine-75 and glycine-76 (Ub-2GA) with alanines. Cell-surface biotinylation assays confirmed maturation and delivery of both parental and chimeric receptor forms to the plasma membrane (Fig. 2A). In unstimulated cells EGFR-Y1045F is not ubiquitylated (15), and consistent with previous studies, was found predominantly at the plasma membrane (Fig. 2B). In contrast, EGFR-Y1045F::Ub-G76A localised primarily to structures resembling endosomes. Indeed, co-staining of EGFR-Y1045F::Ub-G76A and the early endosomal marker, EEA1 (16), revealed a highly significant degree of co-localisation (Fig. 2C).

To track endocytosis of EGFR and yet avoid ligand- or antibody-induced internalisation, we used a fluorescently labelled monovalent fragment of an anti-EGFR antibody (528-Fab). Firstly, this analysis left out the possibility that EGFR::Ub chimeras, like some lysosomal enzymes (5,6), reach endosomal structures directly from the biosynthetic pathway. Second, when cells were pre-incubated on ice with 528-Fab and then transferred to 37°C, we noted different kinetics of internalisation: unlike

EGFR-Y1045F, which remained largely at the cell surface and started appearing in intracellular vesicles only after 20 minutes (Fig. 2D), some chimeric receptors translocated into endosomes already 5 minutes after transfer to 37°C, and their endocytosis peaked at 10 minutes. In line with different endocytic behaviour, metabolic labelling showed the chimeras to be degraded considerably more rapidly than EGFR-Y1045F in the absence of EGF (Fig. 2E). These results imply that mono-ubiquitylation is sufficient for internalisation of EGFR, and together with the data presented in Figure 1 they suggest that Cbl-mediated tagging of monomeric ubiquitins sorts active receptors to degradation.

Decoration of EGFR with mono-ubiquitins, not poly-ubiquitins, is sufficient for ligand-induced receptor degradation—The observed difference between c-Cbl's activity in isolation (mono-ubiquitylation) and in cells (multi- or poly-ubiquitylation), implies recruitment of a collaborating activity. Hence, we compared ubiquitylation of EGFR in the presence of recombinant E1 and E2, or a crude mixture of cellular factors (namely: reticulocyte lysate). Analysis under electrophoretic conditions aimed at resolving ubiquitylated species, detected a significantly higher ladder of EGFR when incubated with c-Cbl and reticulocyte lysate (Fig. 3A). The smeary pattern was particularly detectable by anti-phosphotyrosine antibodies, indicating selectivity to active receptors. Furthermore, replacing wild type ubiquitin with a Ub-K0 did not change the ubiquitylation pattern (Fig. 3B). Conceivably, Cbl-induced mono-ubiquitylation of phosphorylated EGFRs is followed by recruitment of unknown effectors, which mediate additional mono-ubiquitylation.

To resolve whether EGFR ubiquitylation in cells entails monomeric or polymeric ubiquitin, we utilised a series of ubiquitin mutants with individual lysine-to-arginine substitutions at known sites for chain branching in vivo [lysines 11, 29, 48 and 63; (1,17)]. Preliminary analyses indicated that the ectopic ubiquitin attained a >10-fold excess over the endogenous molecule 18 hours after transfection. Under these conditions all four mutants, namely K11R, K29R, K48R and K63R, reconstituted similar patterns of ligand-induced EGFR ubiquitylation as wild-type ubiquitin (Fig. 3C, and data not shown). Furthermore, no mutant inhibited or delayed EGF-induced degradation of EGFR, and hence, none of the tested lysines appears to be involved in ubiquitin chain branching. To consolidate this conclusion, reciprocal experiments were performed using Ub-K0. When over-expressed, this mutant acts as a terminator of ubiquitin polymerisation. However, Ub-K0 expressing cells effectively incorporated the mutant form of ubiquitin into EGFR molecules, which retained their normal ubiquitylation pattern (Fig. 3C). As expected, when tested in conjunction with beta-catenin, a well-characterised substrate of poly-ubiquitylation and proteasomal degradation, Ub-K0 abolished the typical ladder of ubiquitylated beta-catenin (data not shown). Moreover, add-back mutants derived from Ub-K0 underwent comparable conjugation to EGFR, and even though they limited the extent of receptor degradation compared to WT-Ub, none extended or enhanced the ladder of ubiquitylated EGFRs (Fig. 3C, and data not shown). In conclusion, because all lysine mutants of ubiquitin coherently generated a pattern consistent with multi-ubiquitylation, these results reinforce a role for mono-ubiquitin in sorting EGFR to endocytosis.

Multi-ubiquitylation confined to the kinase domain of EGFR is sufficient for receptor degradation—The results presented suggest that multi-ubiquitylation of EGFR is preceded by a mono-ubiquitylating event. In an attempt to map the putative site, we generated a truncation mutant containing lysines only within its kinase domain (EGFR-ΔC4R; Fig. 4A), a region essential for c-Cbl recruitment (12). In vitro, EGFR-ΔC4R, like wild-type EGFR, underwent c-Cbl-mediated mono-ubiquitylation (Fig. 4B), raising the possibility that the kinase domain may be specifically targeted by c-Cbl. Testing EGFR-ΔC4R in living CHO cells lent support to this assignment. First, the EGFR-ΔC4R mutant, like the wild-type receptor, underwent weak mono-ubiquitylation in unstimulated cells (Fig. 4C). Further, regardless of its smaller number of potential acceptor sites, EGFR-ΔC4R underwent EGF-induced multi-ubiquitylation and its degradation was accelerated by EGF and c-Cbl (Fig. 4C). Subsequent kinetic experiments, which are not presented, showed that both forms of EGFR underwent comparably rapid ubiquitylation, in support of the possibility that a kinase domain lysine serves as a founder mono-ubiquitylation site.

Discussion

Fusion of a single ubiquitin to different integral membrane proteins led to the conclusion that ubiquitylation controls cargo endocytosis (11,18,19). Our work extends this notion to RTKs and envisages a step-wise process leading to the termination of growth factor signalling: ligand-induced phosphorylation of EGFR recruits c-Cbl (15), and then an E2 molecule, which is physically attached to c-Cbl, discharges its thioester-bonded ubiquitin at a lysine residue, likely located within the kinase domain of EGFR (Fig. 4). Because in isolation c-Cbl conjugates monomeric ubiquitins (Fig. 1B), and covalent attachment of a single ubiquitin drives endocytosis of an internalisation-defective receptor (Fig. 2), we propose that the founder ubiquitin undergoes no branching, and its conjugation instigates sorting of ubiquitylated EGFRs. Predictably, the sorting mechanism involves adaptors bearing UIMs, such as Eps15 and epsin (7,10). Conceivably, while at the cell surface or en route to the lysosome, EGFR is further decorated with additional monomers of ubiquitin. The mechanism of this secondary multi-ubiquitylation step remains unknown.

Considering the ability of a single ubiquitin to drive receptor endocytosis, it is worthwhile asking why EGFR is multi-ubiquitylated? For one, multiple monomers may confer resistance to inhibition by deubiquitylating enzymes. Alternatively, successive mono-ubiquitylation may increase the avidity of EGFR binding to adaptors like Eps15 (10). Last, multi-ubiquitylation rather than poly-ubiquitylation may confer to EGFR refractoriness to the 26S proteasome. Notably, both endocytic adaptors (20) and one of the ubiquitin-binding proteasomal subunits (21) utilise UIMs to recognise their cargoes and substrates. Hence, in the endosomal pathway, the intrinsic inability of c-Cbl to attach more than one ubiquitin to EGFR and to subsequently polymerise ubiquitin, may suffice the endocytic machinery while evading degradation by proteasomes. Consistent with a mechanism that evades the

proteasome, c-Cbl undergoes self-ubiquitylation with no associated proteasomal degradation (22). Hence, the E3 ligase activity of c-Cbl may be confined to mono-ubiquitylation. This issue and the possibility that other E3 ligases complement the action of c-Cbl towards the EGFR are matters for future investigation.

Legends to figures

FIG. 1. **In cells c-Cbl promotes conjugation of several ubiquitins to each EGFR molecule, but in isolation only monomers of ubiquitin are conjugated by c-Cbl.** *A,* CHO cells expressing EGFR, HA-Ub and c-Cbl, as indicated, were incubated without or with EGF (100 ng/ml) for 15 min at 37°C, and cell lysates analysed with the indicated antibodies. *B,* EGFR isolated from untreated A431 cells was subjected to an in vitro ubiquitylation in the presence of E1 and E2 (UbcH5C), together with $(His)_6$HA-Ub, either wild type (WT) or K0, and GST-Cbl, as indicated.

FIG. 2. **Covalently-attached mono-ubiquitin is sufficient to promote endocytosis and degradation of EGFR.** *A, Top,* representation of EGFR-Y1045F::Ub. *Bottom*, EGFR-expressing CHO cells were surface-biotinylated on ice and analysed with the indicated antibodies. *B,* EGFR-expressing CHO cells were fixed, permeabilised and incubated with an anti-EGFR antibody, followed by a Cy3-labelled secondary antibody. *C,* Cells expressing EGFR-Y1045F::Ub-G76A were fixed, permeabilised and co-incubated with antibodies against EEA1 and HA. Secondary fluorescent antibodies were used for detection. *D,* HeLa cells expressing the indicated EGFR forms were pre-incubated for 90 minutes on ice with a Cy3-labelled 528-Fab. Subsequently, cells were incubated at 37°C for the indicated intervals, fixed and analysed. *E,* CHO cells expressing EGFR-Y1045F (circles) or EGFR-Y1045F::Ub-G76A (squares) were subjected to metabolic labelling with ^{35}S-labelled amino acids for 12 hours. EGFR was immunoprecipitated following the indicated chase intervals. Shown are average decay curves and an autoradiogram of a representative experiment.

FIG. 3. **EGFR undergoes multi-ubiquitylation, not poly-ubiquitylation.** *A,* EGFR immunoprecipitates from untreated A431 cells were ubiquitylated in vitro in the presence of either recombinant E1 and E2, or reticulocyte lysate (RL), together with $(His)_6$HA-Ub, and GST-Cbl, as indicated. Reaction mixtures were incubated at 30°C for 60 min before immunoblotting. *B,* In vitro ubiquitylation of isolated EGFRs was performed in the presence of reticulocyte lysate. GST-Cbl and HA-Ub were added as indicated. *C, Top,* representation of all lysines of ubiquitin. Forward (lysine-to-arginine) and add-back (arginine-to-lysine) mutants are depicted. *Bottom,* CHO cells expressing c-Cbl and EGFR, along with the indicated forms of HA-Ub, were incubated without or with EGF for 15 min at 37°C, and cell lysates were analysed as indicated.

FIG. 4. **Cbl-induced ubiquitylation impinges on the tyrosine kinase domain of EGFR.** *A,* Schematic representation of EGFR-ΔC4R, a truncated receptor (residues 1 to 1087) in which lysine residues not included within the tyrosine kinase (TK) domain were mutated. *B,* EGFRs derived from unstimulated transfected HEK-293T cells were subjected to ubiquitylation in vitro in the presence of recombinant E1

and E2. *C,* CHO cells expressing HA-Ub, c-Cbl, and either wild type EGFR (WT) or the truncation mutant (ΔC4R), were analysed as indicated.

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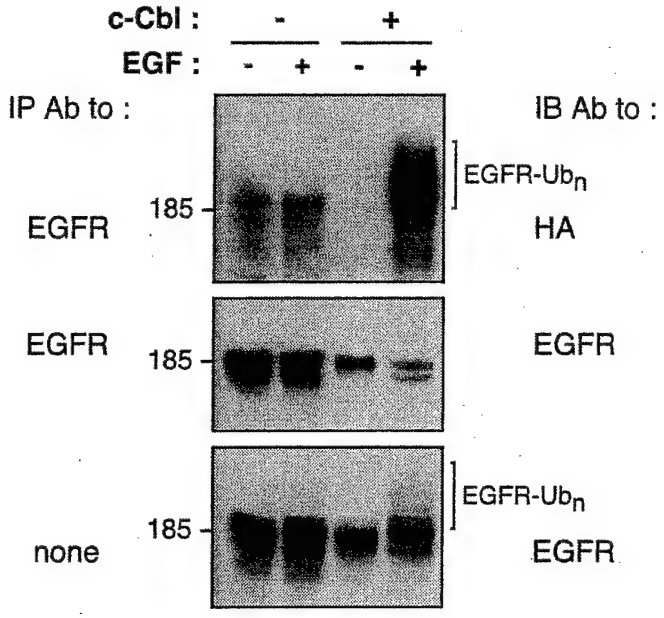
A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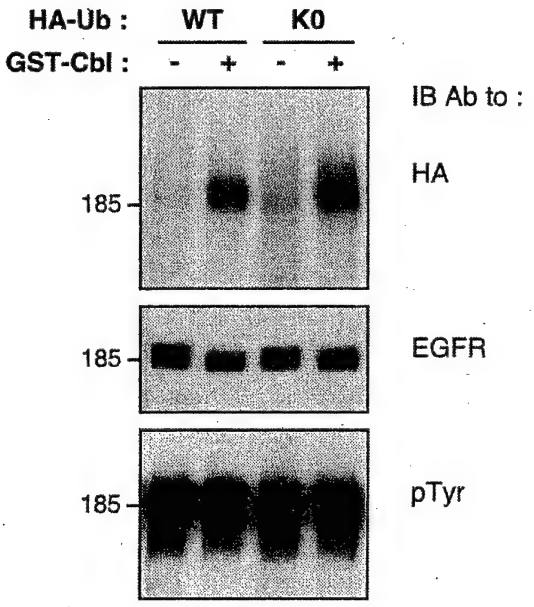
EGFR

B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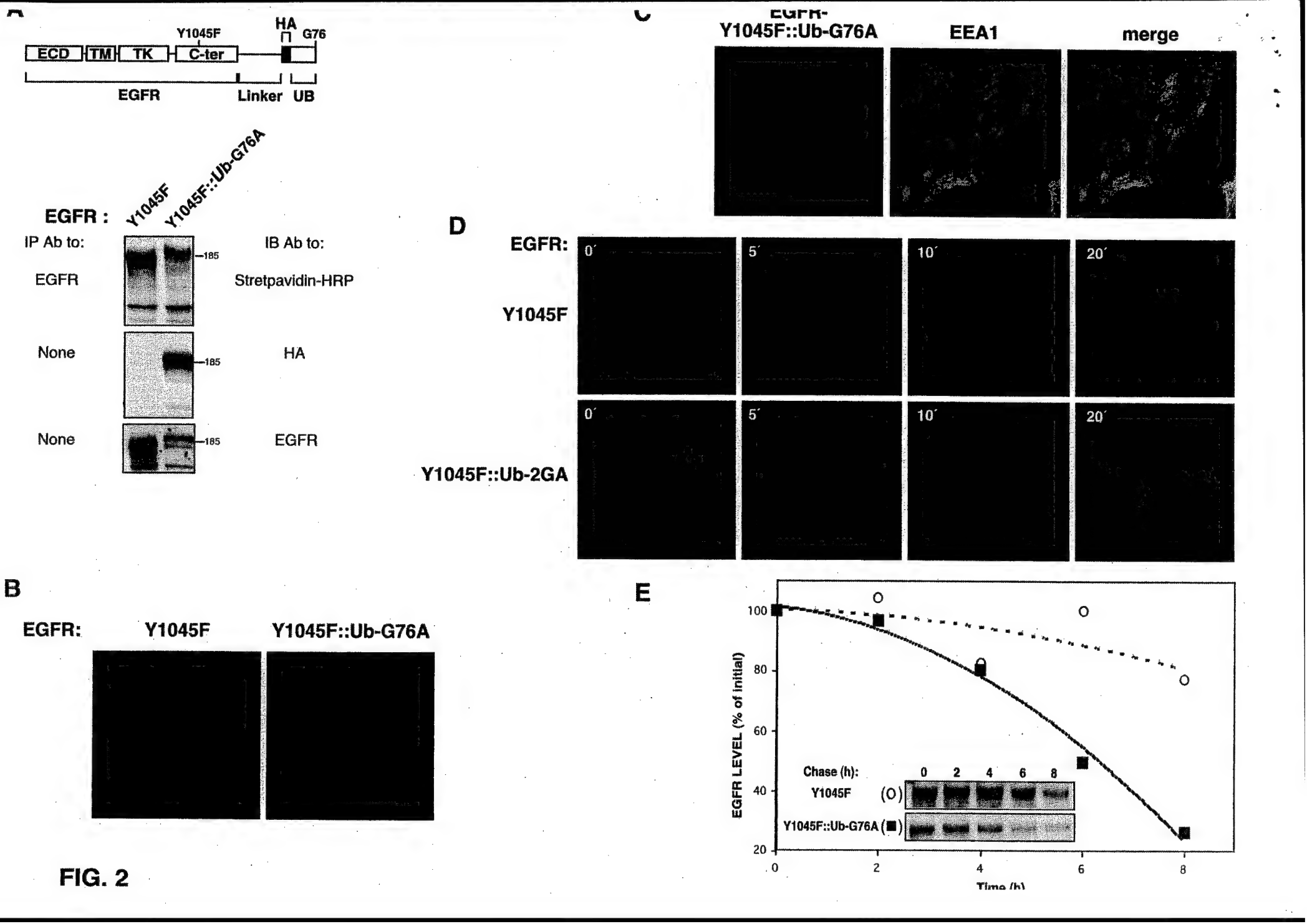
185
HA
185
EGFR
185
pTyr

FIG. 1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G76
ECD
TM
TK
C-ter
EGFR
Linker
UB
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IP Ab to:
IB Ab to:
EGFR
Stretpavidin-HRP
None
HA
None
EGFR
185
C
EGFR-
Y1045F::Ub-G76A
EEA1
merge
D
EGFR:
Y1045F
Y1045F::Ub-2GA
0´
5´
10´
20´
B
EGFR:
Y1045F
Y1045F::Ub-G76A
E
EGFR LEVEL (% of initial)
100
80
60
40
20
0
2
4
6
8
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Chase (h):
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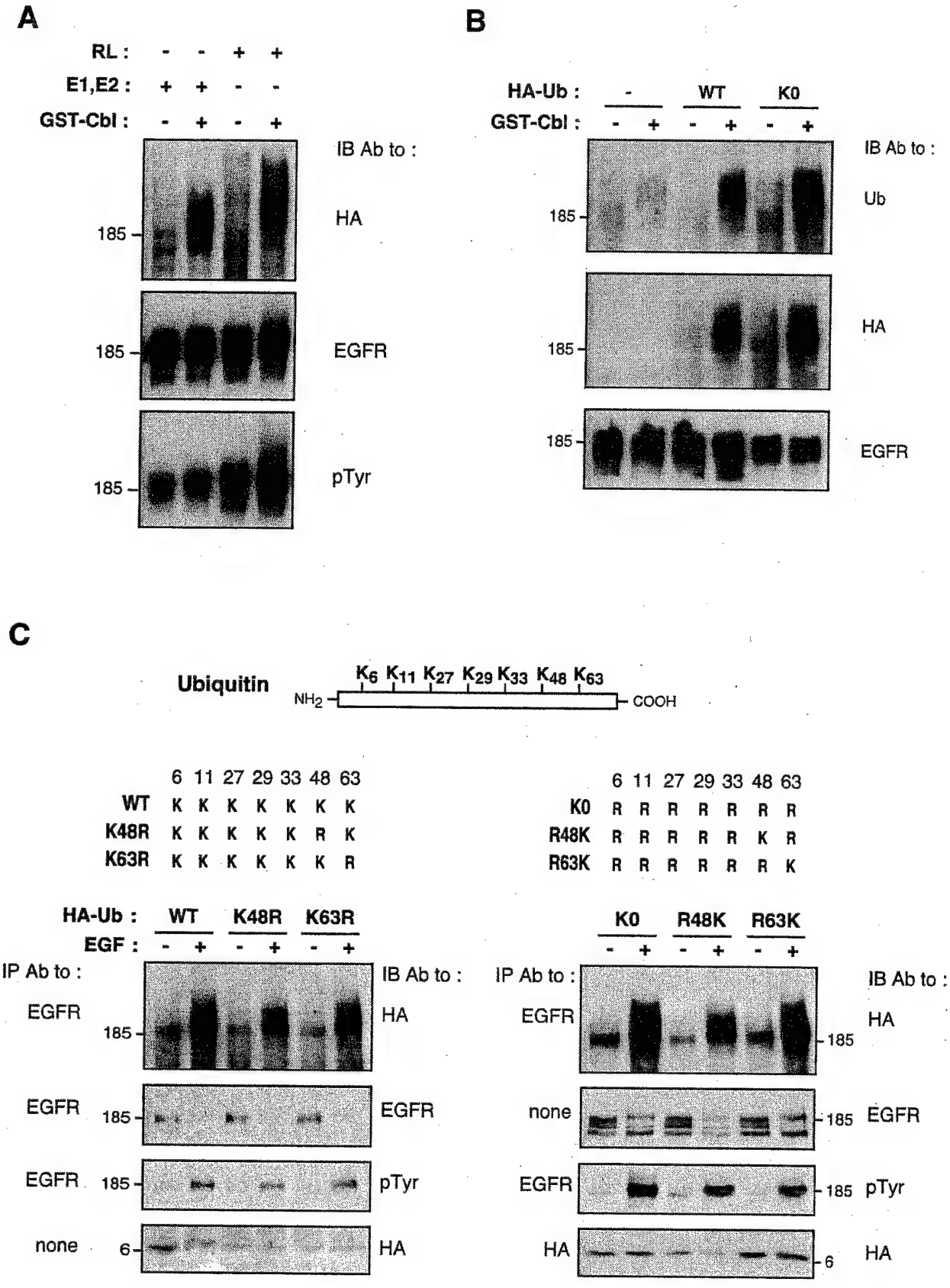
2
4
6
8
Y1045F
Y1045F::Ub-G76A

FIG. 2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GST-Cbl : - + - +
IB Ab to :
HA
EGFR
pTyr
185
B
HA-Ub : - WT K0
GST-Cbl : - + - + - +
IB Ab to :
Ub
HA
EGFR
185
C
Ubiquitin
K6 K11 K27 K29 K33 K48 K63
NH2
COOH
6 11 27 29 33 48 63
WT K K K K K K K
K48R K K K K K R K
K63R K K K K K K R
K0 R R R R R R R
R48K R R R R R K R
R63K R R R R R R K
HA-Ub : WT K48R K63R
EGF : - + - + - +
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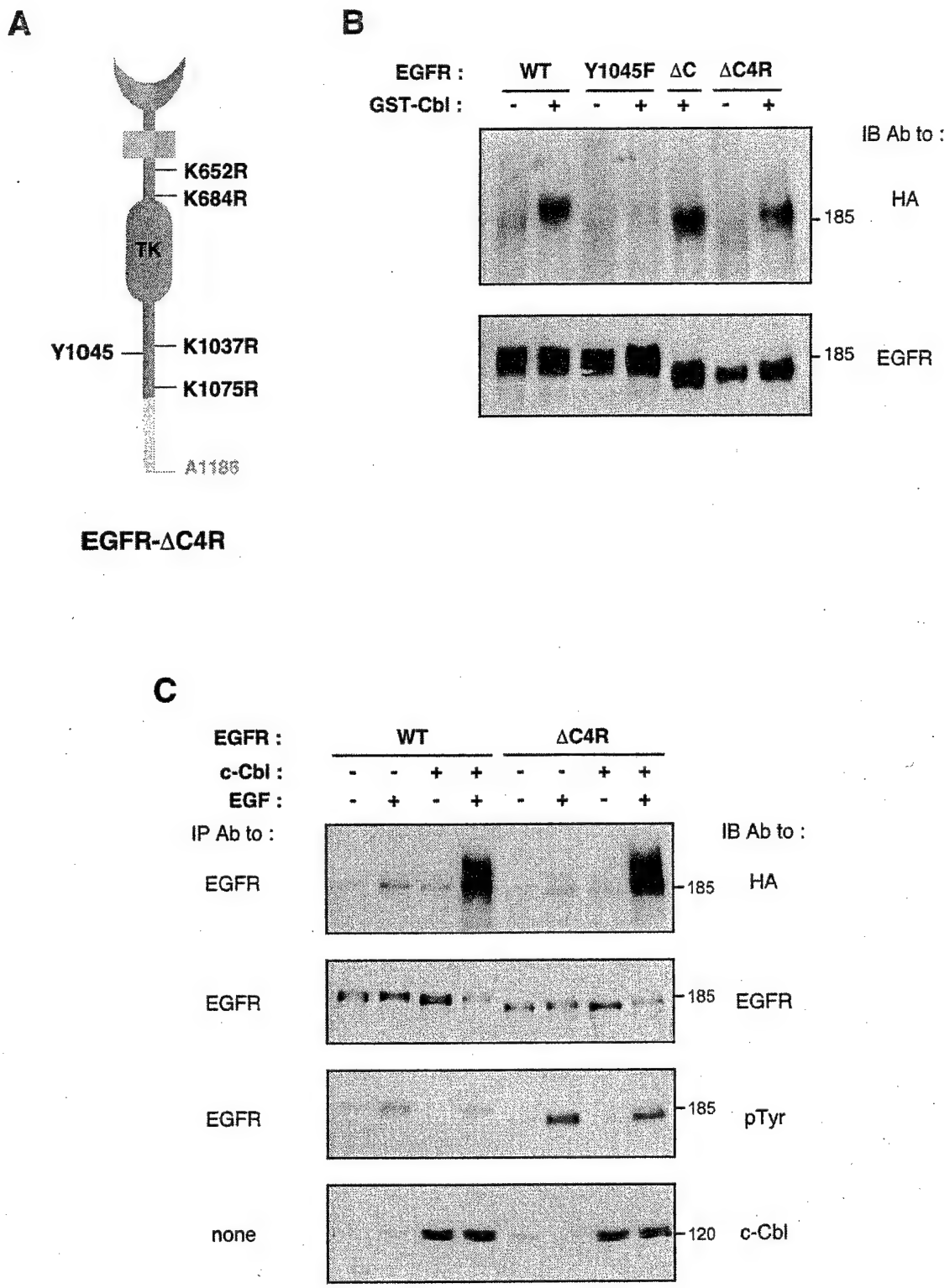
pTyr
6

FIG. 3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B
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ΔC
ΔC4R
GST-Cbl :
- + - + + - +
IB Ab to :
HA
185
185
EGFR
C
EGFR :
WT
ΔC4R
c-Cbl :
- - + + - - + +
EGF :
- + - + - + - +
IP Ab to :
IB Ab to :
EGFR
185
HA
EGFR
185
EGFR
EGFR
185
pTyr
none
120
c-Cbl

FIG. 4

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Ligand-Independent Degradation of Epidermal Growth Factor Receptor Involves Receptor Ubiquitylation and Hgs, an Adaptor Whose Ubiquitin-Interacting Motif Targets Ubiquitylation by Nedd4

Menachem Katz[1,†], Keren Shtiegman[1,†], Pazit Tal-Or[1], Liat Yakir[1], Yaron Mosesson[1], Daniel Harari[1], Yossi Machluf[1], Hironobu Asao[2], Thomas Jovin[3], Kazuo Sugamura[2] and Yosef Yarden[1,*]

[1] Department of Biological Regulation, The Weizmann Institute of Science, Rehovot 76100, Israel
[2] Department of Microbiology and Immunology, Tohoku University School of Medicine, 2–1 Seiryo-machi, Aoba-ku, Sendai 980–77, Japan
[3] Max Planck Institute for Biophysical Chemistry, Am Fassberg 11, D-37077 Gottingen, Germany
** Corresponding author: Yosef Yarden, yosef.yarden@weizmann.ac.il*

Ligand-dependent endocytosis of the epidermal growth factor receptor (EGFR) involves recruitment of a ubiquitin ligase, and sorting of ubiquitylated receptors to lysosomal degradation. By studying Hgs, a mammalian homolog of a yeast vacuolar-sorting adaptor, we provide information on the less understood, ligand-independent pathway of receptor endocytosis and degradation. Constitutive endocytosis involves receptor ubiquitylation and translocation to Hgs-containing endosomes. Whereas the lipid-binding motif of Hgs is necessary for receptor endocytosis, the ubiquitin-interacting motif negatively regulates receptor degradation. We demonstrate that the ubiquitin-interacting motif is endowed with two functions: it binds ubiquitylated proteins and it targets self-ubiquitylation by recruiting Nedd4, an ubiquitin ligase previously implicated in endocytosis. Based upon the dual function of the ubiquitin-interacting motif and its wide occurrence in endocytic adaptors, we propose a ubiquitin-interacting motif network that relays ubiquitylated membrane receptors to lysosomal degradation through successive budding events.

Key words: endocytosis, epidermal growth factor, Hgs/Hrs, Nedd4, signal transduction, tyrosine kinase, ubiquitin

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Desensitization of ligand-activated receptors for growth factors plays a pivotal role in signal transduction, and its deregulation may lead to neoplastic transformation [reviewed in (1,2)]. Regulated endocytosis through clathrin-coated areas of the plasma membrane is the primary process underlying the desensitization mechanism. Lessons learned by studying yeast membrane proteins suggest that sorting of receptors to endocytosis and to subsequent degradation in the vacuole, the yeast counterpart of the lysosome, is controlled by ubiquitylation of both the internalizing receptors and components of the endocytic machinery (3,4). In the case of the epidermal growth factor receptor (EGFR), ligand-induced sorting to lysosomal degradation involves recruitment of a ubiquitin ligase, c-Cbl, to a specific tyrosine residue located at the carboxyl terminal tail of EGFR, and concurrent assembly of a complex comprising an ubiquitin-loaded E2 enzyme (5–8). As in yeast, hydrophobic signals intrinsic to the appended ubiquitin molecule may be sufficient for sorting of receptors to degradation in mammalian cells (9). In addition, a juxtamembrane-localized leucine-based motif has been implicated in internalization of EGFR (10), but the relationships between intrinsic internalization motifs (e.g. di-leucine) and inducible sites (i.e. a specific phosphotyrosine) that mediate endocytosis of EGFR are currently unclear.

Along with rapid ligand-induced endocytosis, a simultaneous constitutive process of slow internalization and subsequent degradation of EGFR takes place in living cells. This process may be exemplified by the internalization capability of a kinase-defective mutant of EGFR (11–13), and it seems to be shared by cargo receptors for vitamins and nutrients. The mechanisms underlying constitutive endocytosis, its dependency on clathrin-coated pits and the role of ubiquitylation in this process are currently unknown. It is relevant, however, that Eps15, an endocytic adaptor that binds to the clathrin complex, and regulates EGFR endocytosis (14), undergoes ligand-induced ubiquitylation (15). Several other adaptors, like Hgs (formerly called Hrs) and members of the STAM family [reviewed in (16)] share a coiled-coil domain, which allows Hgs–STAM interactions. By binding to clathrin (17), phosphatidyl inositol 3′ phosphate (PI3P) (18), and Eps15 (19), Hgs recruits clathrin to early endosomes and disrupts the association of AP-2, the di-leucine binding component of the clathrin-coated pit, with Eps15. Thus, a multimolecular complex comprising Eps15, Hgs and STAM may exist in cells and control receptor endocytosis through regulated interactions with the AP-2/clathrin machinery.

Several structural and functional lines of evidence provide further evidence for the participation of Hgs in receptor endo-

[†] The contribution of these authors was equal.

cytosis. The early endosomal antigen 1, which is involved in endocytic membrane fusion, shares with Hgs a PI3P-binding FYVE domain (18), and localization to endosomes (20,21). Moreover, the probable yeast ortholog of Hgs, Vps27p, belongs to the class E vacuolar protein sorting group, which is involved in membrane traffic through the prevacuolar/endosomal compartment (22). Accumulation of endocytosed proteins, similar to the class E phenotype of yeast, was observed in mammalian cells lacking Hgs expression (23). Interestingly, over-expression of Hgs leads to similar endosomal accumulation of cargo proteins (17,21,24,25). However, it is currently unclear how Hgs is involved in EGFR endocytosis.

To address the endocytic function of the Hgs-coordinated multiprotein complex, we made use of a series of Hgs mutants. Our results attribute to Hgs a role in both the inducible and the ligand-independent pathways of receptor endocytosis. Interestingly, we find that the constitutive pathway is associated with receptor polyubiquitylation and translocation to Hgs-containing endosomes. Unlike receptor ubiquitylation, which positively drives endocytosis, we report that ubiquitylation of Hgs negatively modulates this process. Ubiquitylation of Hgs depends on its UIM, an ubiquitin binding motif that enables recruitment of Nedd4, an E3 ubiquitin ligase whose yeast homolog is widely implicated in endocytosis of membrane proteins. These results are in line with several recent reports on the function of UIM-containing adaptors like Epsin, Eps15, Hgs, and their yeast counterparts in endocytosis (26–29).

Results

A FYVE mutant of Hgs unveils a function in endocytosis of both occupied and unoccupied EGF receptors

To analyze the role of Hgs in endocytosis of EGFR, we attempted to generate an interfering mutant. Figure 1(A) presents the domain structure of Hgs. Because impairment of the FYVE domain resulted in loss of function of Vps27p, the yeast homolog of Hgs (22), we generated three mutants in the FYVE domain (30). Mutations were introduced at two of the conserved cysteines of the domain (mutants denoted C166A and C215A), and in the basic motif surrounding cysteine^{182}, a putative PI3P binding pocket (mutant denoted C-3). In addition, we used the dC2 mutant (31), which is mislocalized (24). In preliminary tests we found that dC2 exerted no significant effect on the rate of EGF internalization, but wild-type Hgs led to the disappearance of 50% of surface EGFRs, and each of the three FYVE-mutant Hgs proteins inhibited internalization of a radiolabeled EGF (data not shown). Because ligand-induced ubiquitylation sorts EGFRs to endocytosis and degradation (32,33), we tested the effect of C166A on EGF-induced ubiquitylation and degradation of EGFR in receptor-negative Chinese hamster ovary (CHO) cells. This analysis revealed that C166A can inhibit the rate of receptor degradation and the extent of ligand-induced receptor ubiquitylation (Figure 1B), a modification initiated by c-Cbl at the cell surface and progressively enhanced en route to the multivesicular body (34,35).

Next, we tested the prediction that wild-type Hgs is involved in receptor ubiquitylation and degradation. Over-expression of Hgs moderately decreased receptor levels in a ligand-independent manner, and the remaining receptors displayed constitutive polyubiquitylation (Figure 1C). These ligand-independent activities require PI3P binding, because FYVE mutants displayed impaired ability to induce receptor degradation, and their effect on basal ubiquitylation of EGFR was smaller than the effect of wild-type Hgs. In contrast to the FYVE domain, deletion of the coiled coil and part of the proline- and glutamine-rich region enhanced EGFR ubiquitylation and degradation (dM mutant; data not shown). By analogy with Hgs, prolonged overexpression of c-Cbl reduced EGFR expression, even in the absence of EGF (Figure 1C), in line with our previous report (6). Nonetheless, upon stimulation by EGF, c-Cbl rapidly enhanced receptor ubiquitylation, and the pattern of this modification displayed higher stoichiometry relative to the effect of Hgs (compare receptor levels and ubiquitin signals in Figure 1C). The relatively slow, ligand-independent effects of Hgs on receptor stability were reflected by two other lines of evidence: metabolic labeling of EGFR followed by a variable chase (in the absence of EGF) revealed that ectopic Hgs shortens the half-life of EGFR from approximately 9.5 h to 6 h (Figure 1D). The alternative approach utilized a hormone-inducible stable expression of Hgs. Treatment of two independent clones with the inducing agent led to Hgs accumulation, and a concomitant reduction in EGFR levels (data not shown), consistent with activation of a slow receptor degradation pathway.

Probing whole cell extracts with a rabbit anti-Hgs antibody detected the endogenous 110-kDa Hgs protein, and also confirmed ectopic expression of Hgs (Figure 1C, lower panel). Interestingly, a ladder of reactive protein bands was detectable when using a polyclonal antibody. These observations suggested that Hgs undergoes ligand-independent ubiquitylation, an issue addressed by our later experiments. In summary, the FYVE domain of Hgs is essential for enhanced ubiquitylation and degradation of both occupied and unoccupied EGFR molecules.

Hgs translocates EGFR to large cytoplasmic vesicles

To understand the mechanism by which FYVE mutants inhibit endocytosis of EGFR, we compared their cellular locations with that of EGFR and an ectopically expressed wild-type Hgs. These experiments made use of a previously described chimeric EGFR fused to GFP (36). When singly expressed in CHO cells, GFP-EGFR displayed primarily cell surface localization (data not shown). Consistent with previous reports (25), over-expression of the wild-type form of Hgs induced the appearance of large, Hgs-containing vesicular structures (Figure 2A), formerly identified as early endosomes [(20) and references therein]. In more than 85% of wild-type Hgs-overexpressing cells a large fraction of GFP-EGFR translocated to Hgs-containing endosomes,

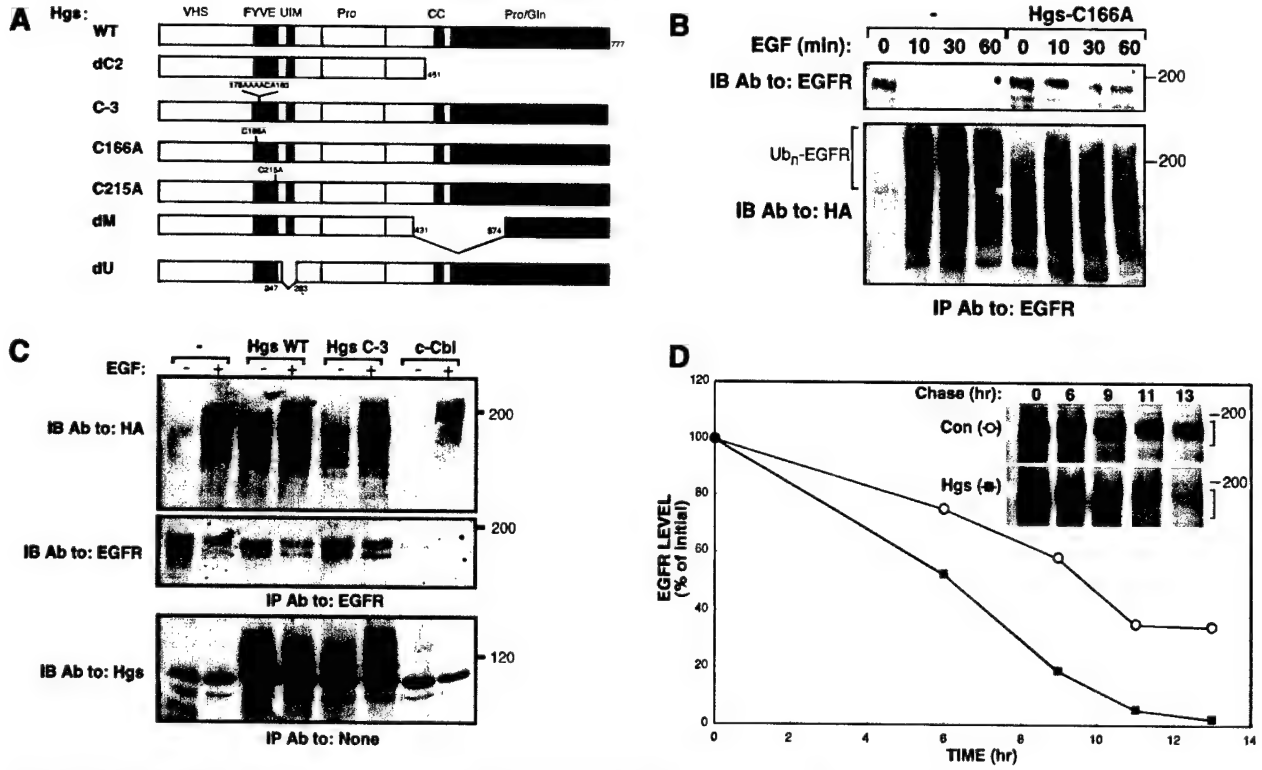

Figure 1: The FYVE domain of Hgs is involved in EGFR ubiquitylation and degradation. *A*, The domain structure of Hgs mutants used in this study is schematically depicted. The following domains are indicated: an N-terminal VHS, a zinc finger FYVE, a UIM, a proline-rich region (*Pro*), a coiled coil domain (*CC*), and a carboxyl terminal proline- and glutamine- rich region (*Pro/Glu*). *B*, CHO cells were cotransfected with an EGFR expression vector and a plasmid encoding HA-tagged ubiquitin, along with a vector encoding a mutant of Hgs (C166A). For control, we used an empty expression vector (lanes labeled -). Forty-eight hours after transfection, the cells were incubated at 37 °C for the indicated time intervals with EGF (100 ng/ml). Whole cell extracts were prepared and subjected to immunoprecipitation (IP) with an anti-EGFR antibody, followed by immunoblotting (IB) with a rat mAb to HA, or a rabbit anti-EGFR antibody. Ubiquitylated forms of EGFR are marked (*Ub$_n$-EGFR*). *C*, CHO cells were cotransfected with an EGFR expression vector and a plasmid encoding HA-tagged ubiquitin, along with plasmids encoding the indicated Hgs proteins or the c-Cbl protein. For control, we used an empty expression vector (–). Forty-eight hours after transfection, cells were incubated at 37 °C for 10 min without or with EGF (100 ng/ml). Cell extracts were subjected to immunoprecipitation (IP) with an anti-EGFR antibody, followed by immunoblotting (IB) with a rat mAb to HA or a rabbit anti-EGFR antibody. The lower panel shows immunoblotting of whole cell lysates with a rabbit anti-Hgs antibody. Note the endogenous p110 Hgs. *D*, COS-7 cells stably expressing HA-Hgs (squares) or untransfected COS-7 cells (control; open circles) were subjected to metabolic labeling with ^{35}S-labeled amino acids for 12 h. Following the indicated time intervals in media containing no radioactivity, cells were extracted and the endogenous EGFR immunoprecipitated. Shown are autoradiograms and the corresponding decay curves calculated after quantifying the marked bands.

independently of EGF binding. In contrast, we observed that dC2 was mislocalized to the periphery of the cell, probably due to the defective proline- and glutamine-rich domain (24), and the distribution of EGFR in dC2-overexpressors was indistinguishable from cells transfected with an empty vector. Receptor translocation requires an intact FYVE, because none of the three FYVE mutants could effectively translocate EGFR (see, for example, the results obtained with C-3, Figure 2A). Unlike wild-type Hgs, these mutants were confined to peripheral aggregates that were largely devoid of EGFR. We concluded that the FYVE domain of Hgs is necessary for translocation of EGFR from the plasma membrane to an endosomal localization.

Several previous studies that tested both structural (37) and functional (38,39) aspects of EGFR internalization concluded that the slower, ligand-independent pathway is undertaken by kinase-defective mutants of EGFR. To relate the Hgs-regulated mechanism, we utilized an EGFR mutant (Kin$^-$) whose ATP-binding site is inactive due to a mutation at lysine 721. Despite lack of catalytic activity, this form of EGFR underwent enhanced ubiquitylation in cells expressing an ectopic Hgs (data not shown). Consistent with these biochemical lines of evidence, morphological studies indicated that wild-type Hgs retained the ability to translocate Kin$^-$ to large Hgs-containing vesicular structures (Figure 2B). Taken together, the results presented in Figures 1 and 2 unveiled the ability of Hgs to

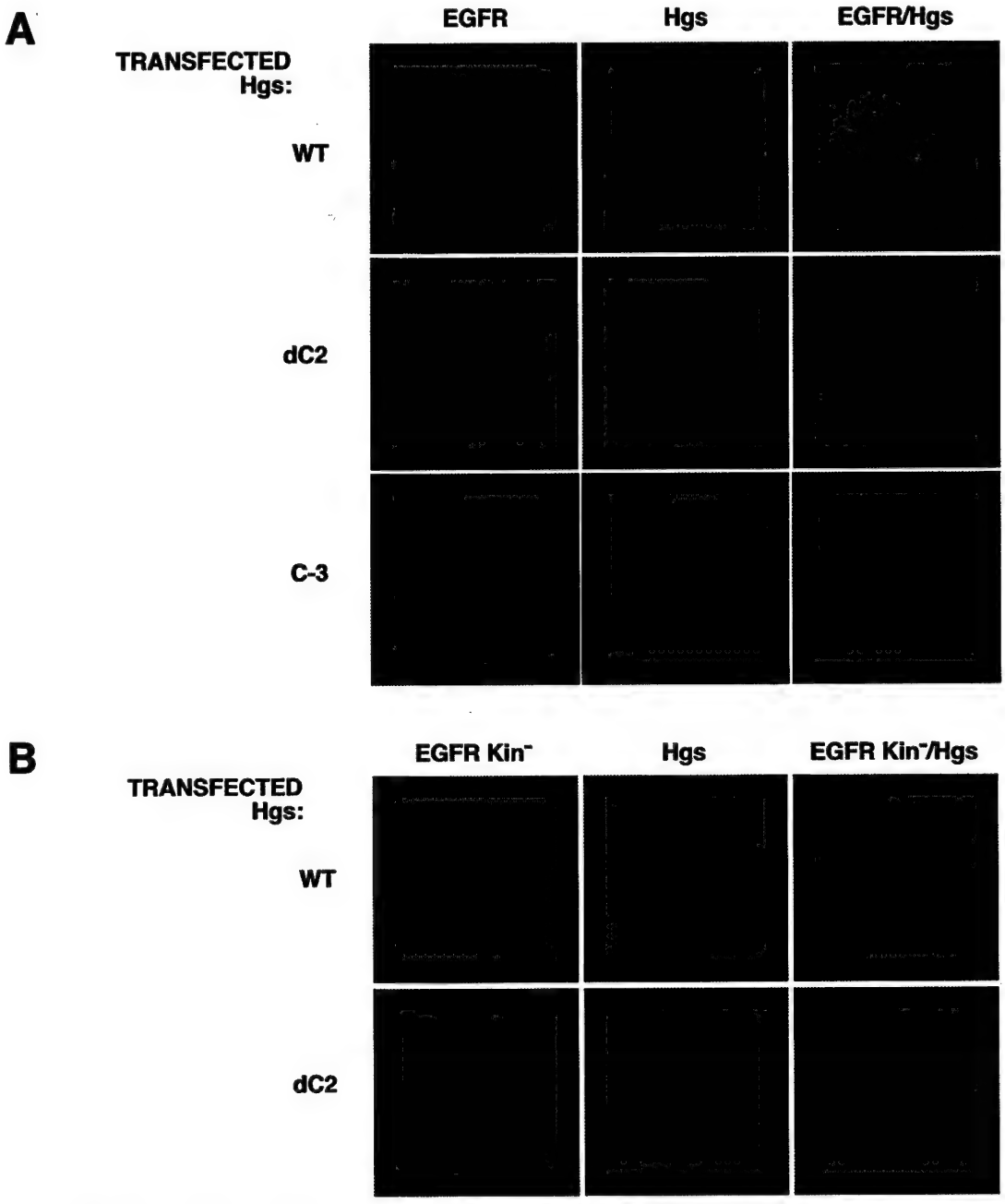

Figure 2: Hgs translocates surface receptors to large cytoplasmic vesicles independently of the tyrosine kinase activity of EGFR. *A*, CHO cells were transfected with expression vectors encoding a full-length EGFR fused to the green fluorescence protein (GFP-EGFR), along with plasmids encoding wild-type (WT) or mutant HA-tagged Hgs proteins (dC2 or C-3). Cells were transferred to cover slips after 1 day, fixed, permeabilized, and incubated with a rat anti-HA mAb. The primary antibody was followed by a Cy3-conjugated anti-rat immunoglobulin G antibody. The fluorescent signal of GFP-EGFR (green) is shown on the left column and the middle column presents the location of Hgs proteins (red). An overlay of GFP and Cy3 fluorescence, generating yellow color in areas of colocalization, is shown in the right column. All panels show middle confocal sections of the cells. *B*, CHO cells transiently expressing a kinase-defective EGFR (EGFR Kin$^-$) along with the indicated HA-tagged Hgs proteins were grown on cover slips for 2 days. The cells were then fixed, permeabilized, and incubated with antibodies that detect HA and EGFR. Cover slips were then incubated with a Cy3-conjugated anti-rat and Cy2-conjugated anti-mouse immunoglobulin G antibodies. The left column presents EGFR staining (green) and the middle column shows Hgs localization (red). An overlay of the two fluorescent signals, generating a yellow color in areas of colocalization, is shown in the right column.

channel EGF-receptors to endocytosis, independently of the receptor's kinase activity.

The UIM of Hgs autonomously mediates self-ubiquitylation

The ladder-like appearance of Hgs in immunoblots (Figure 1C) suggested that this endosomal adaptor undergoes ubiquitylation, reminiscent of the modification of c-Cbl (40) and Eps15 (15). To test this possibility, we coexpressed in CHO cells Flag-tagged ubiquitin and hemagglutinin (HA)-tagged forms of Hgs. The results of this experiment confirmed the existence in living cells of polyubiquitylated forms of Hgs (Figure 3A). Moreover, we demonstrated that Hgs ubiquitylation is independent of the FYVE, coiled-coil or the glutamine- and proline-rich domains. However, deletion of the UIM led to complete abolishment of Hgs ubiquitylation. The existence of a UIM in STAM, a physical partner of Hgs, prompted us to examine whether STAM undergoes ubiquitylation. Co-expression of STAM and a peptide-tagged ubiquitin detected an apparently mono-ubiquitylated form of STAM, but a mutant lacking the UIM displayed low, if any, modification (Figure 3B). Thus, ubiquitylated forms of Hgs and STAM exist in living cells, and the corresponding UIMs are necessary for this protein modification.

Originally identified in S5a, a polyubiquitin binding subunit of the proteasome, the core of the UIM consists of 18 amino acids: an amino-terminal acidic box flanks a stretch of alternating long and short hydrophobic amino acids that terminate at a conserved serine residue [serine 270 (41)]. Because the UIM of Hgs contains no lysine residue, we inferred that this domain targets the ubiquitylation process rather than serving as an ubiquitylation site. To test this hypothesis we individually fused the UIMs of Hgs and STAM, along with short flanking regions, to a modified glutathione S-transferase (GST) protein (mGST). When expressed in CHO cells, the mGST-UIM^{Hgs} and mGST-UIM^{STAM} fusion proteins, respectively, underwent poly- and mono-ubiquitylation (Figure 3C), in line with the corresponding ubiquitylation patterns of full-length Hgs and STAM in living cells. Moreover, mutagenesis of the conserved UIM's serine severely reduced ubiquitylation (Figure 3D), indicating the importance of this residue. Several control mGST proteins containing either no added sequence, or long inserts derived from nonrelevant proteins, displayed no ubiquitylation signals (data not shown). Hence, the results obtained ascribe a novel function to UIMs of endosomal adaptors: these relatively short amino acid sequences autonomously target the machinery responsible for attachment of ubiquitin to substrate adaptor molecules.

The UIM of Hgs binds ubiquitylated proteins and enables Nedd4-mediated ubiquitylation in vitro and in living cells

The tandem UIM of S5a binds polyubiquitylated lysozyme and free polyubiquitin chains (42). To test possible binding of polyubiquitylated proteins to the UIM of Hgs, we used bacterial GST fusion proteins comprising either a combination of the FYVE and UIM domains, or each domain alone (namely GST-FYVE and GST-UIM). The fusion proteins were incubated with extracts prepared from CHO cells expressing HA-ubiquitin-tagged proteins. As expected, recombinant GST-S5a bound many high molecular weight ubiquitylated proteins (Figure 4A), and no ubiquitylated substrates bound to the isolated GST portion. Remarkably, the UIM of Hgs conferred binding to a relatively restricted set of ubiquitylated proteins. The specificity of these interactions was confirmed by lack of binding to the FYVE domain of Hgs or to the SH2 domain of Grb2 (Figure 4A). Noteworthy, the FYVE–UIM fusion protein bound better than the isolated UIM to ubiquitylated substrates. Taken together with the results presented in Figure 3, these observations suggested that the UIM of Hgs is associated with a dual, possibly integrated, function: along with recruiting a ubiquitylation machinery, this domain can bind ubiquitylated substrates.

Next, we addressed the mechanism that enables the UIM to direct protein self-ubiquitylation. By testing two promiscuous E2 ubiquitin-conjugating enzymes, namely UBC-H5C and UBC-H7, we found that the UIM cannot recruit these E2 molecules (data not shown), and therefore it does not qualify as an E3 ligase. Alternatively, the UIM may target an E3 ubiquitin ligase to UIM-containing proteins. Studies in yeast and in mammalian cells implicated Nedd4 in a number of endocytic and exocytic processes [reviewed in (43)]. When tested *in vitro* in the presence of isolated E1 and E2 (UBC-H5C) enzymes, a recombinant bacteria-derived Nedd4 enhanced the ubiquitylation of not only UIM-containing fusion proteins, but also of high molecular weight aggregates, whose identity remains unknown (Figure 4B). Significantly weaker signals were observed in the absence of Nedd4. Likewise, when the E3 ligase was incubated with control fusion proteins lacking the UIM we observed weaker signals, probably because ubiquitylated substrates were not retained by this fusion protein. To complement this *in vitro* line of evidence, we constructed a catalytically inactive mutant of Nedd4, in which the HECT domain cysteine capable of forming a thiolester bond with ubiquitin (44) was replaced by a serine (C853S-Nedd4). When introduced into cells expressing the mGST-UIM^{Hgs}, the catalytically inactive Nedd4 almost abolished polyubiquitylation of the UIM-containing protein (Figure 4C), implying that Nedd4 is a major ubiquitin ligase recruited by UIM^{Hgs}. In line with this conclusion, an ectopically introduced wild-type Nedd4 moderately increased ubiquitylation of mGST-UIM^{Hgs} (Figure 4C), suggesting that Nedd4 is constitutively active in living cells.

To verify the relevance of these findings to full-length Hgs, we examined the effect of ectopic Nedd4 on the level of Hgs ubiquitylation in living cells, and observed increased polyubiquitylation (Figure 4D). Consistent with an essential role for the UIM, Nedd4 exerted minimal or no effect on the state of ubiquitylation of Hgs proteins, which are either devoid of the UIM (dU mutant), or carrying an inactivating mutation (S270D; Figure 4D). Upon verifying expression of mutant Nedd4 and Hgs proteins, we noted differences in levels of expression relative to the respective wild-type proteins. How-

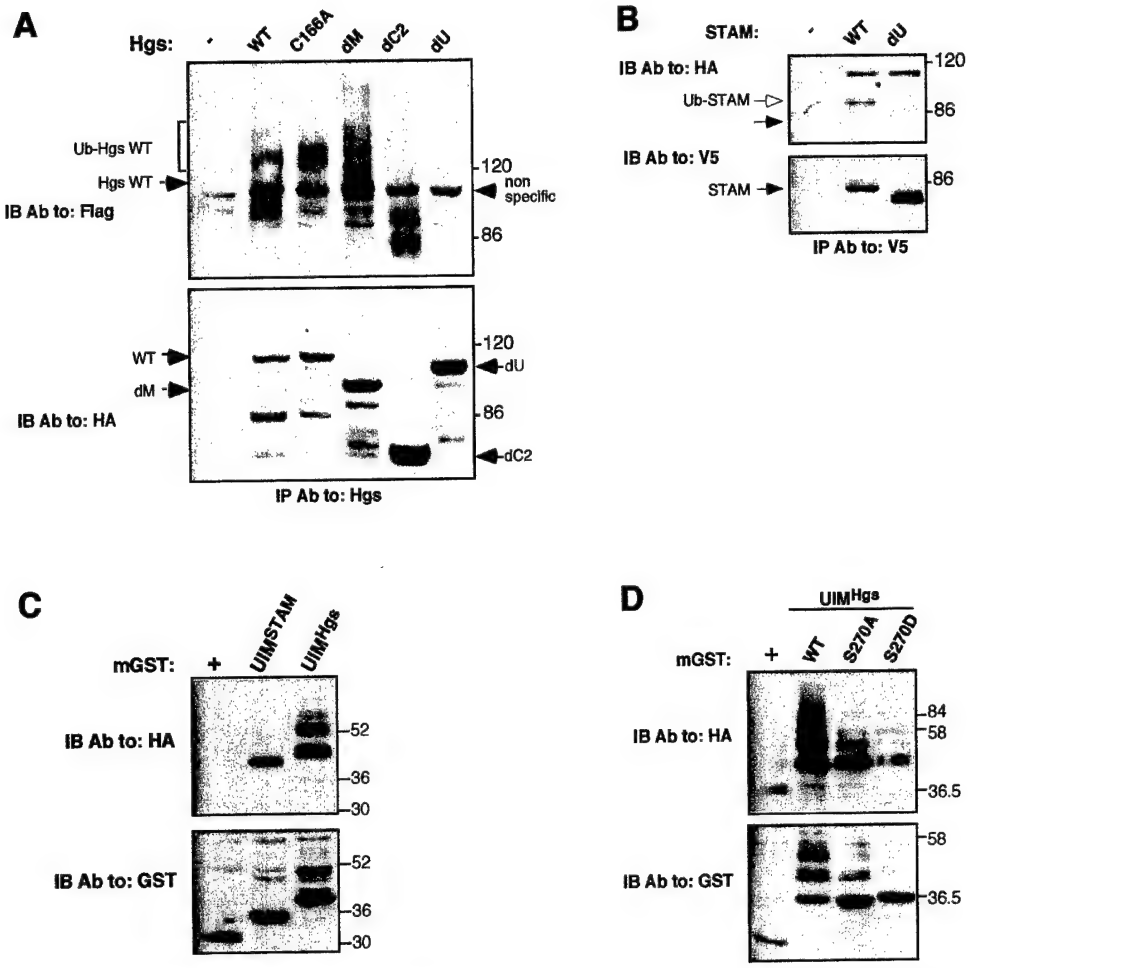

Figure 3: Hgs and STAM are ubiquitylated proteins whose UIM domains autonomously drive self-ubiquitylation. *A*, CHO cells were cotransfected with an expression vector encoding flag-tagged ubiquitin, along with plasmids encoding the indicated HA-tagged Hgs proteins (see Figure 1A) or an empty expression vector (–). Cell extracts were prepared 2 days after transfection, cleared, and subjected to immunoprecipitation (IP) with an anti-Hgs antibody, followed by immunoblotting (IB) with a mouse anti-Flag mAb or a rat anti-HA mAb, as indicated. The locations of ubiquitylated and non-ubiquitylated Hgs molecules are indicated, along with a nonspecific band at the 110 kDa region. *B*, CHO cells were cotransfected with plasmids encoding the indicated V5-tagged STAM proteins and HA-ubiquitin. As a control, we used an empty expression vector (–). Cell extracts were prepared 2 days after transfection and subjected to immunoprecipitation (IP) and immunoblotting (IB) with the indicated antibodies. Filled arrows mark the STAM protein band and an open arrow indicates the ubiquitylated form of STAM. *C*, CHO cells were cotransfected with plasmids encoding HA-tagged ubiquitin, along with vectors driving expression of the UIM domains of STAM or Hgs fused to mGST. For control we used a plasmid encoding mGST alone (+). Forty-eight hours post transfection, whole cell lysates were subjected to a pull-down assay utilizing GSH-agarose beads. Proteins were then separated by gel electrophoresis and immunoblotted with the indicated antibodies. *D*, HEK-293 cells were cotransfected with plasmids encoding HA-tagged ubiquitin and either mGST alone, or mGST fused to either the wild-type UIM domain of Hgs or a domain mutated at the conserved serine residue. Forty-eight hours post transfection, whole cell lysates were subjected to a pull-down assay utilizing GSH-agarose beads. Proteins were then separated by gel electrophoresis and immunoblotted with the indicated antibodies. Note the retarded electrophoretic mobility of ubiquitylated proteins and the weak mono-ubiquitylation of mGST.

ever, additional experiments are needed to determine whether the HECT and UIM regions of Nedd4 and Hgs, respectively, modulate protein stability. Regardless of this possibility, our results establish functional interactions between a HECT-domain ubiquitin ligase, namely Nedd4, and the UIM of Hgs. Conceivably, when present on a substrate protein like Hgs, the UIM specifically directs the catalytic activity of Nedd4, resulting in substrate ubiquitylation.

Ubiquitylation of Hgs inactivates its endocytic function

To test the role of Hgs ubiquitylation in endocytosis and degradation of EGFR, we compared the effects of wild-type Hgs and a mutant lacking the UIM (denoted dU). When co-expressed with EGFR in CHO cells, both forms of Hgs enhanced disappearance of the receptor, but dU was reproducibly associated with a more extensive effect and with the

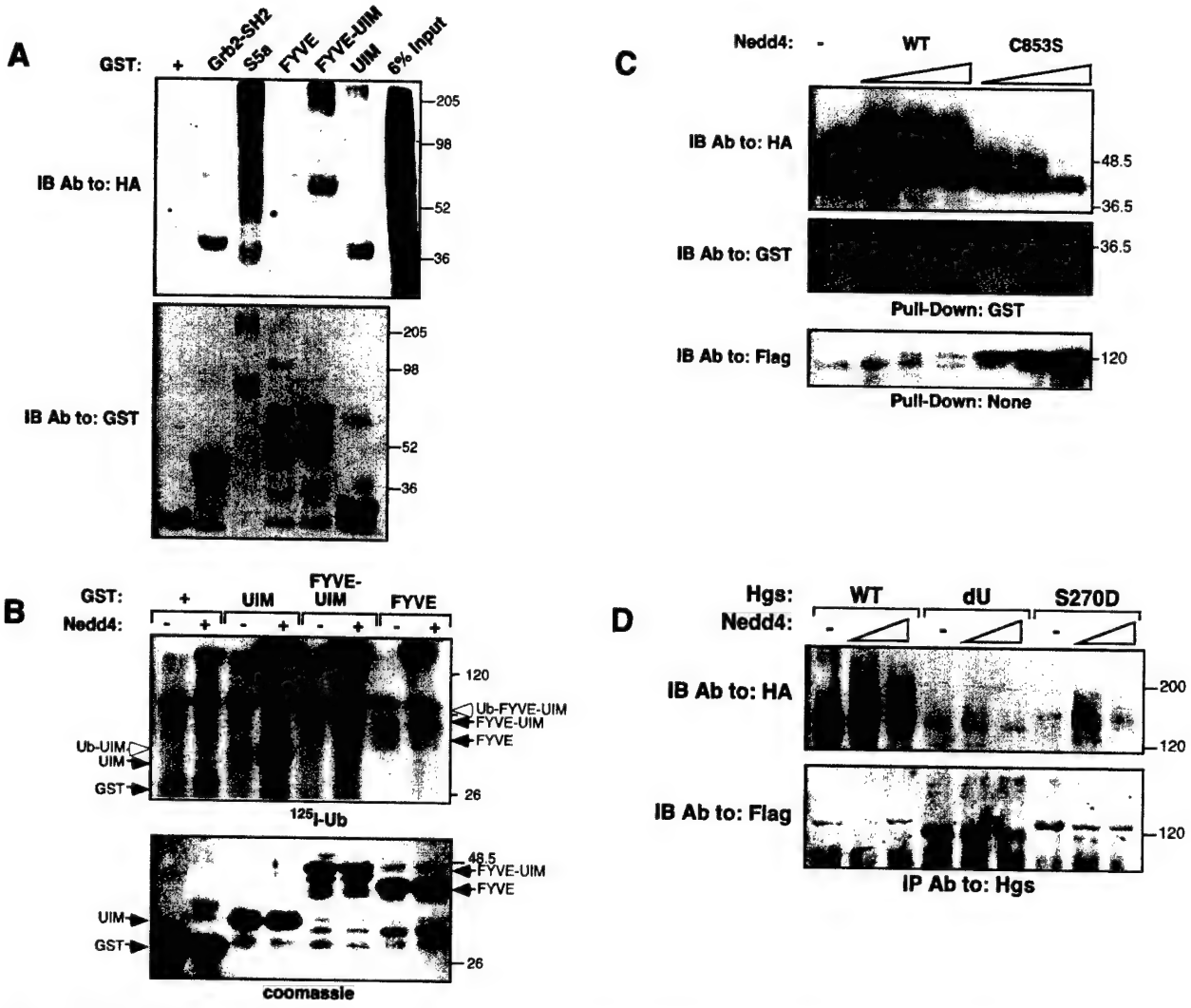

Figure 4: The UIM binds ubiquitylated proteins and autonomously mediates ubiquitylation of Hgs by Nedd4. *A*, CHO cells were transfected with a vector encoding for HA-ubiquitin. Following 48 h, cell extracts were either directly resolved by gel electrophoresis (*Input*; 6% of extract applied to each mixture), or they were subjected to a pull-down assay using the indicated GST fusion proteins (approximately 50 μg each, except for GST-S5a: 10 μg; see lower panel). Gel-separated proteins were immunoblotted with anti-HA or anti-GST antibodies. *B*, The indicated immobilized GST fusion proteins were incubated for an hour at 37 °C with a mixture of a purified E1 enzyme, a recombinant E2 (UBC-H5C), and a radiolabeled ubiquitin, in the absence or presence of an E3 enzyme (recombinant Nedd4 isolated from bacteria). Thereafter, proteins immobilized on the beads were washed and resolved by gel electrophoresis and autoradiography. The lower panel shows a stained gel with the respective GST fusion proteins. Note a nonspecific protein band co-migrating with ubiquitylated FYVE-UIM. *C*, HEK-293 cells were cotransfected with a plasmid encoding for mGST-Hgs-UIM and HA-tagged ubiquitin, along with increasing amounts of expression vectors encoding either a Flag-tagged wild-type (*WT*) Nedd4 or a protein mutated at the catalytic cysteine (*C853S*; 0.2, 0.4, and 1 μg DNA). As a control, we used an empty expression vector (–). Whole cell extracts were subjected to a pull-down assay utilizing GSH-agarose beads, followed by immunoblotting (IB) with the indicated antibodies. The lowest panel shows immunoblotting of whole cell extracts with an anti-Flag antibody. Note the apparent increase in stability of the catalytically inactive mutant of Nedd4. *D*, HEK-293 cells were cotransfected with plasmids encoding HA-tagged ubiquitin, the indicated Flag-tagged Hgs proteins, and increasing amounts of an expression vector encoding Nedd4 (1 and 3 μg DNA). As a control, we used an empty expression vector (–). Whole cell extracts were prepared, cleared and subjected to immunoprecipitation (IP) with an anti-Hgs antibody, followed by immunoblotting (IB) with the indicated antibodies.

appearance of a presumed degradation product (p150; Figure 5A). A similar protein band appeared upon expression of another active mutant of Hgs, namely dM, which is devoid of the coiled-coil and flanking regions. Interestingly, in the presence of dM or wild-type Hgs, the residual receptor displayed more extensive polyubiquitylation than the modification we observed with dU, probably because most ubiquitylated receptors available for endocytosis are already de-

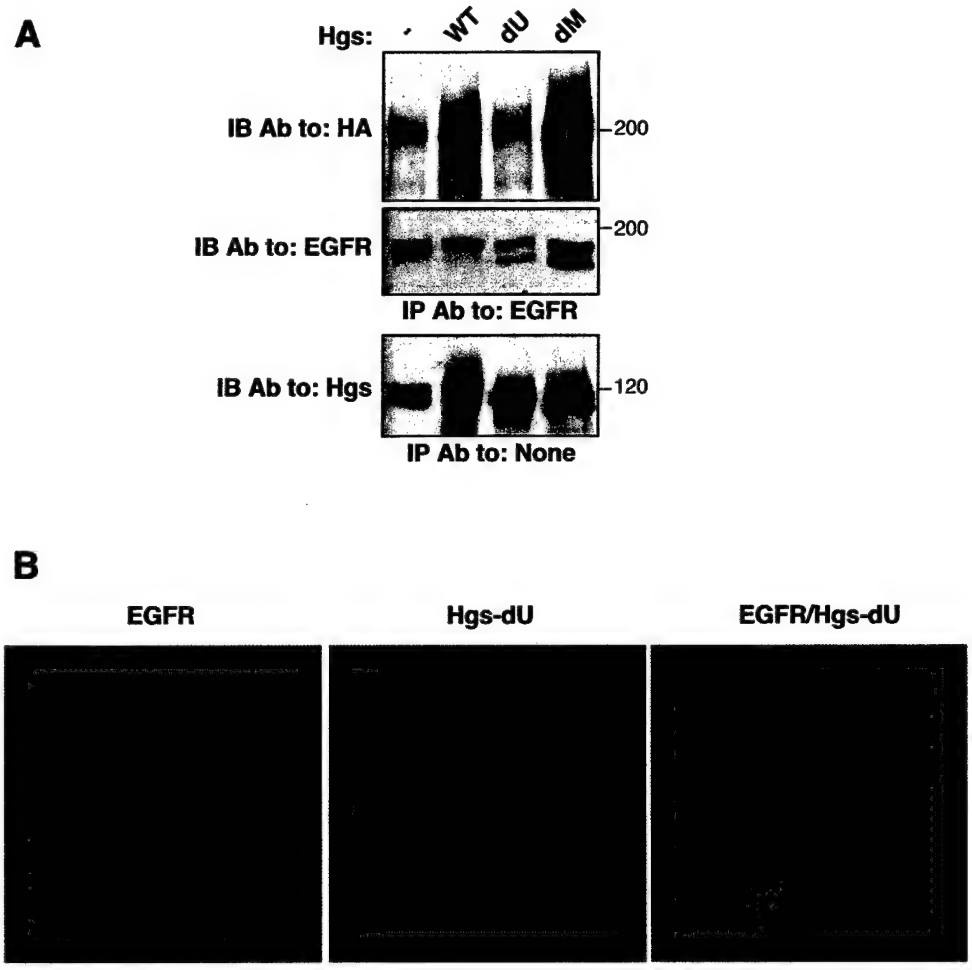

Figure 5: Deletion of the UIM enhances Hgs-induced endocytic degradation of EGFR. *A*, CHO cells were cotransfected with an EGFR expression vector and a plasmid encoding for HA-tagged ubiquitin, along with plasmids driving expression of the indicated Hgs proteins. As a control, we used an empty expression vector (–). Whole cell extracts were prepared and subjected to immunoprecipitation (IP) with an anti-EGFR antibody, followed by immunoblotting (IB) with the indicated antibodies. The lower panel shows immunoblotting of whole cell lysates with an anti-Hgs antibody. *B*, CHO cells were transfected with expression vectors encoding a full-length EGFR fused to the green fluorescence protein (GFP-EGFR), along with a plasmid encoding for an UIM-deleted, HA-tagged Hgs protein (dU). Cells were grown for 2 days on cover slips, fixed, permeabilized, and incubated with a rat anti-HA mAb. The primary antibody was followed by a Cy3-conjugated anti-rat immunoglobulin G antibody. The fluorescent signal of GFP-EGFR (green) is shown on the left column and the middle column presents the location of Hgs proteins (red). An overlay of GFP and Cy3 fluorescence, generating yellow color in areas of colocalization, is shown in the right column. All panels show middle confocal sections of the cells.

graded in dU-expressing cells. Consistent with this scenario, in three out of 12 experiments we observed enhanced ubiquitylation of EGFR in cells expressing dU, and in all experiments receptor degradation was significantly more extensive than in cells expressing similar levels of ectopic wild-type Hgs. Morphological analyses confirmed that dU retained the ability to enhance endocytosis of EGFR (Figure 5B). This mutant, like wild-type Hgs (see Figure 2A), led to translocation of EGFR from the cell surface to vesicular structures, some of which contained Hgs (Figure 5B). Because the UIM is absolutely necessary for ubiquitylation of Hgs (Figure 3A) and this modification is mediated primarily by Nedd4 (Figure 4D), we propose that Nedd4 inactivates the endocytic function of adaptors by elevating their ubiquitylation. Possible functional links between ubiquitin binding to UIMs, consequent targeting of ubiquitin ligases to substrate proteins, and the concerted activities leading to endocytosis of cargo receptors are discussed below.

Discussion

Observations made in yeast established the notion that endocytosis of membrane proteins (e.g. Ste2p) is associated with ubiquitylation of the internalizing protein [reviewed in (4)]. However, because endocytosis of a Ste2p-ubiquitin chimera, a receptor whose internalization does not require ubiquitylation, is defective in cells expressing a mutant E3 ligase,

namely Rsp5p/Nedd4, it has been concluded that ubiquitylation of a component of the endocytic machinery is required for receptor endocytosis (3). By analogy, endocytosis of the growth hormone receptor depends on an intact ubiquitylation machinery, even when the cytoplasmic domain contains no ubiquitylation site (45). Thus, ubiquitylation of both the cargo receptor and a trans-acting target seems involved in endocytosis. Unlike Ste2p and the growth hormone receptor, at least two distinct endocytic pathways are accessible to growth factor receptors harboring an intrinsic tyrosine kinase [reviewed in (1)]. The ligand-induced pathway, which involves receptor auto-phosphorylation, and recruitment of the c-Cbl ubiquitin ligase, has been extensively studied. In the case of EGFR, the much less understood alternative route is often studied by following the internalization of kinase-dead receptor mutants (13,38,39). Understanding this slow, ligand- independent pathway relies on identification of its molecular players. The results we obtained with Hgs identify this endosomal adaptor as one regulator of the constitutive pathway. Along with regulating ubiquitylation of the cargo receptor in a process involving the FYVE domain, the UIM of Hgs autonomously mediates self-ubiquitylation. However, whereas receptor ubiquitylation positively drives endocytosis, UIM-dependent and Nedd4-mediated ubiquitylation of Hgs inactivates its endocytic action.

The role of Hgs in receptor endocytosis and degradation

Several previous reports attributed to Hgs a mostly inhibitory action in ligand-induced endocytosis of different receptors (17,21,26,31,46,47). However, most previous studies were based upon overexpression, which yields a phenotype similar to nullifying Hgs expression (23). In contrast, utilizing FYVE mutants we provide evidence for a positive role of Hgs in both constitutive and ligand-induced endocytosis. Consistent with involvement in the constitutive pathway, it has been reported that Hgs regulates uptake of a fluid phase marker (17), as well as transferrin (19), two well-characterized markers of the constitutive pathway. Our evidence implicates receptor ubiquitylation in the Hgs-associated sorting of EGFRs. Thus, mutant Hgs proteins incapable of elevating receptor ubiquitylation (e.g. C-3) were unable to translocate EGFR from the plasma membrane to endocytic vesicles (Figure 2A), whereas expression of a relatively potent mutant of Hgs (i.e. dM) was associated with extensive ubiquitylation, endocytosis and degradation of EGFR (data not shown). It is therefore plausible that receptor ubiquitylation sorts for degradation of both ligand-occupied and unoccupied receptors.

A network of UIM-containing adaptors that relays ubiquitylated membrane receptors to lysosomal degradation

Our finding that both Hgs and STAM undergo ubiquitylation in living cells (Figure 3) implicates ubiquitylation of an endocytic machinery encompassing at least the two adaptors. The recently uncovered endocytic functions of additional UIM-containing adaptor proteins like Epsin/Ent1p, Eps15/Ede1p and Hgs/Vps27p imply global involvement of protein ubiquitylation in regulating endocytosis of cargoes (26–29). A key issue, however, is the relationship between receptor endocytosis and covalent modification of the endocytic machinery. Unlike Ste2p and the growth hormone receptor, whose endocytosis depends on ubiquitylation of the endocytic machinery (3,45), blocking ubiquitylation of and ubiquitin binding to Hgs enhanced endocytic degradation of EGFR (Figure 5). Hence, UIM-mediated ubiquitylation of Hgs may inhibit, rather than enhance, the function of Hgs in endocytosis. De-ubiquitylation of c-Cbl seems to recycle the ligase following dissociation from an endocytosed receptor (40). In addition, genetic analyses identified functional interactions between Epsin, a UIM-containing endosomal protein, and a de-ubiquitylating enzyme required for normal eye development in flies (48). In support of a positive endocytic role of de-ubiquitylating enzymes, genetic and biochemical evidence from yeast implies that another de-ubiquitylating enzyme, Doa4p, is necessary for sorting membrane proteins and lysosomal hydrolases to the vacuole (49,50).

By concentrating on the UIM of Hgs and STAM, we showed that this relatively short domain is endowed with two autonomous activities: not only can it bind polyubiquitylated proteins (Figure 4A), but grafting a UIM onto a protein targets the fusion chimera for ubiquitylation (Figure 3C), most likely by Nedd4 (Figure 4C,D). In line with this conclusion, the UIM of Eps15 (27), as well as the UIM of the yeast counterparts of Hgs and Epsin (29), is endowed with a dual function. Importantly, ubiquitylation by Nedd4 modifies regions outside of the UIM and, in the context of Hgs, this protein modification may restrict, rather than enhance degradation of internalized receptors (Figure 5). Are these seemingly distinct activities of the modular UIM domain linked in some way? Ubiquitylation of Hgs and other adaptors may alter protein conformation, or it may generate new docking sites, which can lead to auto-catalyzed nucleation of large multiprotein complexes. According to an alternative mechanism, UIM-containing adaptors are active in their non-ubiquitylated form, namely: they can bind an ubiquitylated receptor either at the cell surface (e.g. the UIM of Epsin) or at endosomal compartments (e.g. the UIM of Hgs). On the other hand, once ubiquitylated by Nedd4, the UIM-containing adaptor is inactivated, because the appended ubiquitin blocks the UIM, thereby unloading the ubiquitylated receptor. The mechanism we propose is analogous to inactivation of $pp60^{Src}$ through binding of a carboxyl terminal phospho-tyrosine to an intrinsic SH2 domain (51).

The results we presented on Hgs (Figure 1B) and previous data on Eps15 (14) indicate that UIM-containing proteins control both the rapid, ligand-induced pathway of EGFR endocytosis, as well as the tonic endocytosis taking place in the absence of EGF. Likewise, the UIM of Hgs shunts internalized chimeras of Ub-transferrin receptor to lysosomal degradation (28), and *Drosophila* embryos lacking Hgs fail to degrade active EGFR molecules (26). Hence, one shared rate-determining step for endocytic pathways may be the speed of receptor ubiquitylation at the plasma membrane and in endo-

some membrane invaginations. Whereas c-Cbl is the ligase responsible for ubiquitylation of ligand-occupied EGFR molecules at the plasma membrane (33), the identity of the putative cargo-specific E3 ligase that assists Hgs is still unknown. Another open question relates to EGF-inducible phosphorylation of UIM-containing adaptors like Hgs, STAM and Eps15. Our observation that STAM undergoes mono-ubiquitylation (Figure 3B), similar to the observed modification of Eps15 (27), but Hgs is a substrate for polyubiquitylation (Figure 3A), raises the issue of variable ubiquitin branching. These and other aspects of the proposed mode of action of the UIM require additional studies.

Materials and Methods

Reagents and antibodies

Unless indicated, materials were purchased from Sigma (St. Louis, MO, USA). Radioactive materials were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK) and IODOGEN from Pierce (Rockford, IL, USA). Lipofectamine was supplied by Gibco BRL (Grand Island, NY, USA). G418 was purchased from Calbiochem (San Diego, CA, USA). An anti-HA rat monoclonal antibody (mAb) was purchased from Roche Molecular Biochemicals (Mannheim, Germany). mAbs SG565 and 111.6 to EGFR were generated in our lab. An mAb and a rabbit polyclonal antibody to Hgs were previously described (31). An anti V-5 mouse mAb was purchased from Invitrogen (Groningen, the Netherlands). Antibodies to GST and EGFR were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Peroxidase-conjugated and fluorescently labeled antibodies were purchased from Jackson ImmunoResearch (West Grove, PA, USA). GST-S5a and ubiquitin activating enzyme E1 were from Affiniti (Mumhead, Exeter, UK). Peroxidase-conjugated protein-A was from ICN (Costa Mesa, CA, USA). A chemiluminescence kit for immunoblotting was obtained from Amersham (Buckinghamshire, UK). Protein G Sepharose beads were from Pharmacia Biotech (Buckinghamshire, UK).

Buffers

The following buffers were used: HNTG: 20 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol. TBST: 20 mM Tris-Hcl (pH 7.5), 0.15 M NaCl, and 0.05% Tween 20. Solubilization buffer: 50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 30 mM β-glycerol phosphate, 0.2 mM Na_3VO_4 and a protease inhibitor cocktail (diluted at 1 : 1000).

Construction of expression vectors

The pcDNA3 plasmid (Invitrogen) was used for expression in mammalian cells. Plasmids encoding Hgs (wild type) and two mutants (dC2 and dM) have been previously described (31). Additional mutations were generated in pcDNA3-Hgs by site-directed mutagenesis using the QuikChange mutagenesis kit (Stratagene). The C-3 mutant, containing a substitution of the protein motif RKHHCR flanking cysteine^{182} to AAAACA, was generated by overlap extension PCR (30). The UIM deletion mutant (dU, containing a deletion of 35 amino-acids) was similarly constructed. HA-Hgs was subcloned into pIND expression vector utilizing the *Xhol* and *Xbal* sites, to generate an ecdysone-inducible Hgs. An expression vector (pcDNA3) containing a V5-tagged STAM cDNA has been described (31). The dU mutant of STAM (carrying a deletion of 35 amino-acids in between lysine^{162} and tyrosine^{198}) was generated in this vector. A plasmid encoding a flag-tagged ubiquitin (pEF-Ubiquitin-flag) was generated by overlap extension PCR. A vector encoding a fusion protein comprised of a full-length EGFR fused to the green fluorescence protein (GFP) has been described (36). Flag-Nedd4 expression vector was provided by Marius Sudol (Rockefeller University, New York). The ubiquitin-HA expression vector was a gift from Dirk Bohmann (EBI, Heidelberg, Germany). All other expression vectors have been described (6).

Generation of GST fusion proteins

The GST-FYVE-UIM encoding plasmid (18) was provided by Harald Stenmark (Norwegian Radium Hospital, Oslo, Norway). A stop codon was introduced to replace amino acid 231 generating GST-FYVE. The UIM domain of Hgs and its flanking region (amino acids 225–285) was cloned by PCR into pGEX-4T1 for expression in bacteria. Likewise, we cloned the region corresponding to amino acids 225–284 into a modified pEF-BOS vector for expression in mammalian cells. A similar approach was used to generate mGST-UIM-STAM (amino acids 138–197). GST-Nedd4 bacterial expression vector was provided by Allan Weissman (National Institutes of Health, Bethesda). GST-Grb2-SH2 was obtained from Jan Sap (New York University).

Cell culture and transfection

Chinese hamster ovary cells were cultured in DMEM:F12 medium supplemented with antibiotics, glutamine and heat-inactivated fetal calf serum (10%). For transfection, cells were grown to 80% confluence in 100-mm plates. Transfection was performed using the Lipofectamine method. The following amounts of DNA were used: EGFR and its mutants, 1 μg; HA-ubiquitin, 1 μg; c-Cbl, 3 μg; and Hgs and its mutants, 5 μg. The total amount of DNA in each transfection was normalized with the pcDNA3 plasmid. Cells were assayed 48 h after transfection. COS-7 cells stably expressing HA-Hgs were cultured in G418 (700 μg/ml) containing medium.

Immunoprecipitation, pull-down and immunoblotting analyses

Transfected cells were washed briefly with ice-cold saline, scrapped in solubilization buffer and incubated on ice for 20 min. Lysates were cleared by centrifugation (10 000 × g, 20 min). In experiments where EGF was introduced, the transfected cells were washed in serum-free medium, and incubated for 10 min at 37 °C with EGF (100 ng/ml). For direct electrophoretic analysis, gel sample buffer was added to cell lysates. For equal gel loading, protein concentrations were determined prior to immunoprecipitation by using the Bradford technique. For immunoprecipitation, lysates were incubated for 1–2 h at 4 °C with antibodies precoupled to anti-mouse IgG- agarose beads, or to protein G-agarose beads. The immunoprecipitates were washed thrice with HNTG solution, resolved by gel electrophoresis, and electrophoretically transferred to a nitrocellulose membrane. Membranes were blocked in TBST buffer containing 10% low-fat milk, blotted with a primary antibody for 1 h, washed with TBST and incubated for 30 min with a secondary antibody linked to horseradish peroxidase. Immuno-reactive bands were detected using chemiluminescence. For pull-down assays, cell lysates were incubated for 2 h at 4 °C with glutathione-agarose beads, and tightly bound proteins separated by gel electrophoresis.

Immunofluorescence

Transfected cells grown on cover-slips were fixed for 15 min with 3% paraformaldehyde in PBS. Fixed cells were washed in PBS and permeabilized for 10 min at 22 °C with PBS containing 1% albumin and 0.2% Triton X-100. For labeling, cover-slips were incubated for 1 h at room temperature with an anti-HA antibody, either alone or in combination with the anti-EGFR mAb 111.6. After extensive washing in PBS, the cover-slips were incubated for an additional hour with a Cy3-conjugated donkey anti-rat $F(ab)_2$ alone, or in combination with a Cy2-conjugated donkey anti-mouse $F(ab)_2$. The cover-slips were mounted in mowiol (Calbiochem). Confocal microscopy was performed using a Zeiss Axiovert 100 TV microscope (Oberkochen, Germany) with a 63X/1.4 plan-Apochromat objective, attached to the Bio-Rad Radiance 2000 laser scanning system, operated by LaserSharp software.

In vitro ubiquitylation assay

Glutathione S-transferase fusion proteins (4 μg per reaction) were immobilized on glutathione-agarose beads, washed and incubated in buffer containing 25 mM Tris HCl, 6 mM $MgCl_2$, 100 mM NaCl, 2 μM DTT, 0.2 mM ATP and ^{125}I-Ubiquitin (0.5 μg per reaction). Whole cell extracts (100 μg) or purified E1 (160 ng), E2 (UBC-H5C; 7 μl of crude bacterial extract) and Nedd4 (100 ng; produced in bacteria as a GST fusion protein and cleaved with thrombin to release GST) were added as indicated. Reaction mixtures were incubated for 1 h at 37°C. The beads were then extensively washed, and GST-fusion proteins were eluted with gel sample buffer and resolved by gel electrophoresis.

Metabolic labeling of cultured cells

COS-7 cells were incubated for 14 h in cysteine- and methionine-free medium supplemented by 0.1 mCi/ml ^{35}S-labeled amino acids. Cells were then washed thoroughly and incubated in media containing nonlabeled cysteine and methionine for the indicated time intervals (chase). This was followed by cell lysis, immunoprecipitation, electrophoresis and autoradiography.

Acknowledgments

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A mutant EGF-receptor defective in ubiquitylation and endocytosis unveils a role for Grb2 in negative signaling

Hadassa Waterman, Menachem Katz, Chanan Rubin, Keren Shtiegman, Sara Lavi, Ari Elson[1], Thomas Jovin[2] and Yosef Yarden[3]

Departments of Biological Regulation and [1]Molecular Genetics, The Weizmann Institute of Science, Rehovot 76100, Israel and [2]Department of Molecular Biology, Max Planck Institute for Biophysical Chemistry, Am Fassberg 11, D-37077 Gottingen, Germany

[3]Corresponding author
e-mail: yosef.yarden@weizmann.ac.il

Ligand-induced desensitization of the epidermal growth factor receptor (EGFR) is controlled by c-Cbl, a ubiquitin ligase that binds multiple signaling proteins, including the Grb2 adaptor. Consistent with a negative role for c-Cbl, here we report that defective Tyr1045 of EGFR, an inducible c-Cbl docking site, enhances the mitogenic response to EGF. Signaling potentiation is due to accelerated recycling of the mutant receptor and a concomitant defect in ligand-induced ubiquitylation and endocytosis of EGFR. Kinetic as well as morphological analyses of the internalization-defective mutant receptor imply that c-Cbl-mediated ubiquitylation sorts EGFR to endocytosis and to subsequent degradation in lysosomes. Unexpectedly, however, the mutant receptor displayed significant residual ligand-induced ubiquitylation, especially in the presence of an overexpressed c-Cbl. The underlying mechanism seems to involve recruitment of a Grb2 c-Cbl complex to Grb2-specific docking sites of EGFR, and concurrent acceleration of receptor ubiquitylation and desensitization. Thus, in addition to its well-characterized role in mediating positive signals, Grb2 can terminate signal transduction by accelerating c-Cbl-dependent sorting of active tyrosine kinases to destruction.

Keywords: growth factor/SH2 domain/signal transduction/tyrosine kinase/ubiquitin ligase

Introduction

Polypeptide growth factors mediate cell-to-cell interactions by initiating an ordered cascade of membranal and cytoplasmic events culminating in altered gene expression (van der Geer *et al.*, 1994). While events involved in signal generation and maintenance are extensively characterized, our understanding of processes that terminate signaling is relatively limited. The role for such negative signaling pathways extends beyond the ability to terminate intracellular signals. For example, analyses of one of the major signaling pathways, the mitogen-activated protein kinase (MAPK) cascade, led to the realization that the amplitude and duration of MAPK activation critically determine not only the kinetics but also the identity of cellular responses to hormonal signals (Marshall, 1995). The ErbB family of growth factor receptors exemplifies the importance of negatively acting regulatory pathways and their significance to human diseases (reviewed in Yarden and Sliwkowski, 2001). The four ErbB proteins bind a large group of growth factors all sharing an epidermal growth factor (EGF) domain. Interestingly, the four receptors differ in their signaling potency in accordance with distinct mechanisms that negatively regulate the receptor's fate. For example, only ErbB-1 (also called EGFR) is strongly coupled to the c-Cbl adaptor protein, and this receptor, unlike other ErbB members, is effectively targeted to lysosomal degradation (Levkowitz *et al.*, 1998). Similarly, the ortholog of ErbB proteins in *Caenorhabditis elegans*, LET-23, is negatively regulated by SLI-1, the ortholog of mammalian Cbl proteins (Jongeward *et al.*, 1995). Recent studies that made use of an *in vitro* ubiquitylation system uncovered the role of c-Cbl as an E3 ubiquitin ligase that recruits ubiquitin-loaded E2 enzymes to ligand-activated receptors (Joazeiro *et al.*, 1999; Levkowitz *et al.*, 1999; Waterman *et al.*, 1999; Yokouchi *et al.*, 1999). Apparently, Cbl proteins bind ligand-activated receptor tyrosine kinases through their N-terminally located phosphotyrosine-binding domain, whereas the flanking RING finger enables close apposition of an E2 enzyme, permitting transfer of ubiquitin to target proteins.

Exactly how c-Cbl-induced poly-ubiquitylation of EGFR regulates delivery to the lysosome remains an open question. Internalization of yeast membrane proteins is initiated by protein mono-ubiquitylation (reviewed by Hicke, 2001). In line with the possibility that a similar mechanism operates in mammalian cells, internalization of the macrophage growth factor receptor is retarded in c-Cbl-defective cells (Lee *et al.*, 1999). However, although overexpression of c-Cbl enhanced ubiquitylation of EGFR, no concurrent increase in receptor internalization rate could be demonstrated (Levkowitz *et al.*, 1998; Thien *et al.*, 2001). Likewise, it is unclear whether c-Cbl is recruited to EGFR at the plasma membrane or only when the receptors reach the early endosome (Levkowitz *et al.*, 1998; Lee *et al.*, 1999; Stang *et al.*, 2000). To address the function of c-Cbl in negative signaling, we made use of a mutant EGFR, the c-Cbl docking site of which—Tyr1045—was defective (Levkowitz *et al.*, 1999). Here we provide evidence that this site restricts the mitogenic action of EGFR by enabling recruitment of c-Cbl to the plasma membrane-localized receptors, thereby accelerating their internalization and delivery to lysosomes. By employing the defective receptor, we uncovered a secondary pathway that allows indirect coupling of c-Cbl to activated receptors. This surrogate pathway involves the

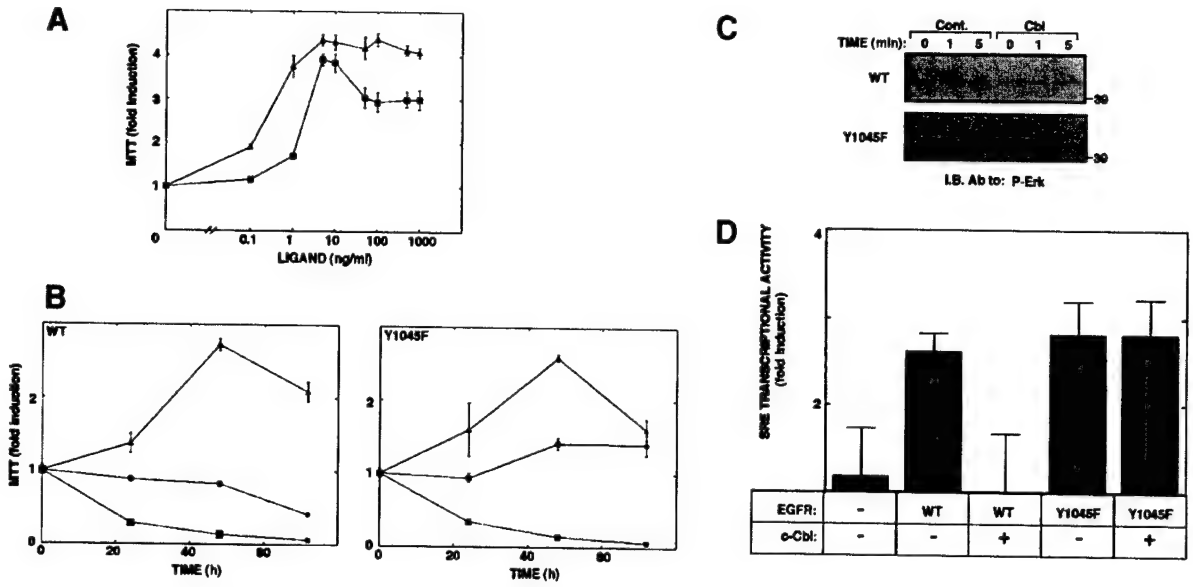

Fig. 1. A mutant EGFR defective at Tyr1045 elicits potent signals and is refractory to c-Cbl. (**A**) Sublines of 32D cells that express either a wt-EGFR (squares), or a Tyr1045 mutant (Y1045F; triangles) were deprived of IL-3 and plated at a density of 5×10^5 cells/ml in media containing serial dilutions of EGF. The MTT assay was performed 24 h later. The results obtained are presented as fold induction relative to a control culture maintained in the absence of added growth factor. (**B**) The indicated sublines of 32D cells (5×10^5 cells/ml) were incubated for various time intervals with EGF at 100 ng/ml (circles). Control cultures were incubated in the absence (squares) or presence of IL-3 (triangles). Cell growth was determined daily by using the colorimetric MTT assay, and compared with the signal observed at time zero. The data presented are the mean ± SD of four determinations. (**C**) CHO cells were transiently transfected with plasmids encoding either a wt-EGFR (WT) or a Tyr1045 mutant (Y1045F). Alongside, we used a vector encoding c-Cbl or a control empty vector. Cell monolayers were stimulated with EGF (100 ng/ml) for the indicated time intervals at 37°C. Whole-cell lysates were analyzed with an antibody specific to the active form of MAPK. (**D**) CHO cells were co-transfected in triplicates as in (C), along with a reporter plasmid (SRE-luc). Thirty-six hours later cells were untreated or treated with EGF (20 ng/ml). Following an additional 12 h, cells were harvested for a luciferase assay. Signals obtained were normalized to protein concentrations and are presented as average ± SD.

adaptor function of Grb2. Thus, our studies help resolve the role of c-Cbl in receptor desensitization and reveal a previously uncharacterized negative function of Grb2.

Results

An EGFR mutant defective at Tyr1045 elicits stronger mitogenic signals

Replacement of Tyr1045 of EGFR with a phenylalanine reduced ligand-induced down-regulation in living cells and significantly reduced receptor ubiquitylation (Levkowitz *et al.*, 1999). To resolve the relationship between receptor ubiquitylation and signaling capacity, we stably expressed the mutant receptor (Y1045F) in interleukin-3 (IL-3)-dependent 32D myeloid cells. These cells are originally devoid of any ErbB protein, but ectopic expression of ErbB receptors confers mitogenic responsiveness to the respective ligands. Cell lines expressing comparable numbers of wild-type and mutant receptors were established by drug selection and their growth examined using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay. When cultured for 24 h in the absence of IL-3, but in the presence of increasing concentrations of EGF, cells expressing a wild-type (wt) EGFR generated a moderate mitogenic signal (Figure 1A). However, the mutant receptor defective in c-Cbl binding elicited significantly higher proliferative signals. The relatively potent mitogenic potential of the mutant receptor was also reflected in a long-term cell growth assay. Whereas EGF only slightly extended survival of cells expressing wt-EGFR, ligand stimulation of 32D cells expressing Y1045F-EGFR exerted marked cell proliferation, albeit lower than the effect observed with IL-3 (Figure 1B). To examine the possibility that Y1045F-EGFR is endowed with relatively potent mitogenic signaling because it escapes inhibition by c-Cbl, we tested the effect of an ectopic c-Cbl on signaling downstream of EGFR (Figure 1C and D). The biochemical assays employed were MAPK activation and transcription of a reporter gene controlled by the serum response element (SRE). The assays were performed with Chinese hamster ovary (CHO) cells, because these cells express no endogenous EGFR. As expected, stimulation of wt-EGFR with EGF resulted in marked activation of MAPK, but introduction of an exogenous c-Cbl significantly reduced the effect of EGF. Likewise, transcription from the SRE was markedly elevated by EGF and almost completely suppressed when c-Cbl was overexpressed. The Y1045F mutant mediated comparable MAPK activation, as well as marked transcription from the SRE, but both activities were unaffected by an ectopically overexpressed c-Cbl. Taken together with the results of the cell growth assays, these observations indicate that c-Cbl acts as a suppressor of EGFR signaling.

Abrogation of c-Cbl's interaction with EGFR retards receptor endocytosis and accelerates recycling

To address the relationships between c-Cbl recruitment and receptor endocytosis, we made use of a previously

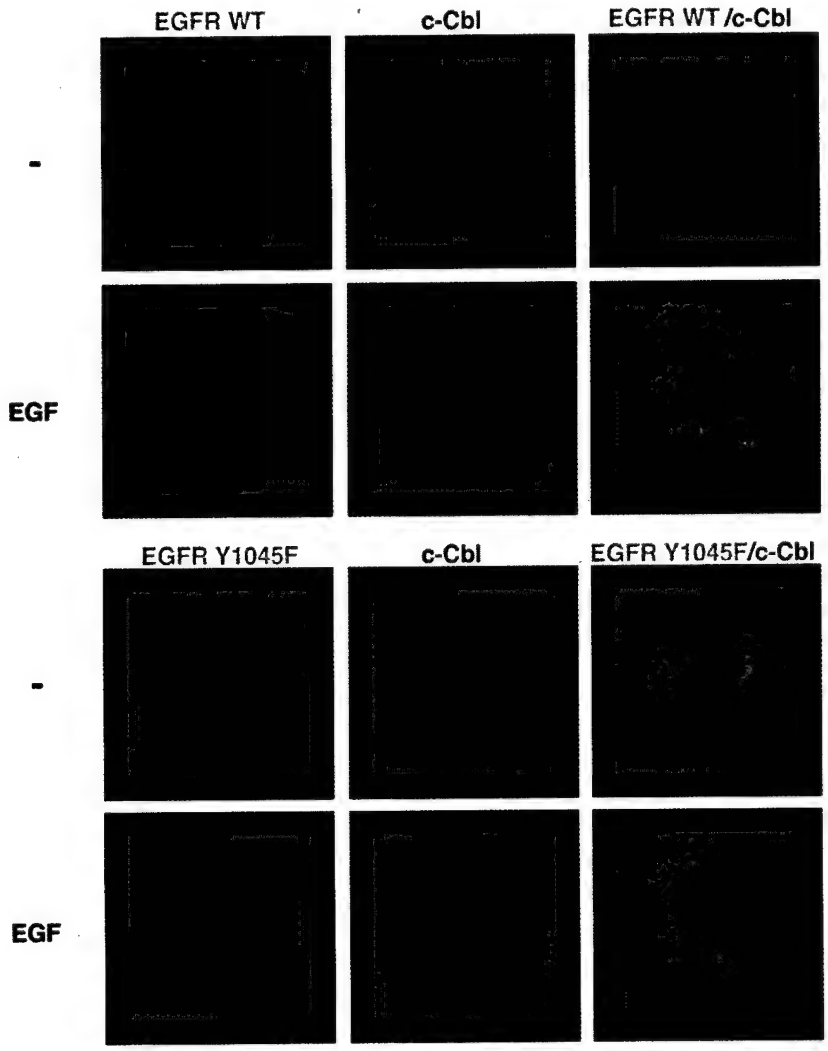

Fig. 2. The c-Cbl docking site of EGFR is necessary for ligand-induced receptor endocytosis and for translocation of c-Cbl to endocytic vesicles. CHO cells transiently expressing HA-tagged c-Cbl along with a wild-type GFP–EGFR (upper panel), or a similar fusion protein containing a mutation at Tyr1045 (Y1045F; lower panel), were grown on cover slips. Cells were incubated for 15 min at 37°C without or with EGF (100 ng/ml). To visualize c-Cbl, cells were fixed, permeabilized and incubated with an anti-HA antibody, followed by incubation with a Cy3-conjugated secondary antibody (red, middle column). The GFP–EGFR fluorescence is represented in the left column (green). The right column presents the overlay of GFP and Cy3 fluorescence, generating a yellow color in areas of co-localization.

described fusion protein comprised of a full-length EGFR fused to green fluorescence protein (GFP) (Brock *et al.*, 1999). A mutation was introduced in the codon corresponding to Tyr1045 and the plasmid used to co-transfect CHO cells. Consistent with previous reports, the plasma membrane-associated wt-EGFR translocated into large endocytic vesicles upon short exposure of cells to EGF (Figure 2). Prior to stimulation with a ligand, c-Cbl was localized primarily to the cytoplasm (Wang *et al.*, 1999). However, shortly after stimulation with EGF, a large fraction of c-Cbl translocated into cytoplasmic vesicles, many of which contained the GFP–EGFR. These results are consistent with observations made with a GFP–Cbl fusion protein (Levkowitz *et al.*, 1998). The behavior of GFP–Y1045F revealed remarkable differences: upon stimulation with EGF, this mutant underwent only limited translocation into endosomal vesicles. Furthermore, in line with a major defect in internalization, the mutant receptor was unable to induce translocation of c-Cbl.

To elucidate the defect in internalization of the Y1045F mutant receptor, we performed several kinetic assays. For a reference mutant whose ligand-induced internalization is defective we used an EGFR devoid of tyrosine kinase activity due to a mutation in the ATP-binding site (K721A) (Chen *et al.*, 1989; Felder *et al.*, 1990). A short-term ligand internalization assay confirmed the relatively slow internalization rate of EGF by K721A (Figure 3A). In the same assay Y1045F displayed an intermediate rate of ligand endocytosis. Likewise, whereas the wt-EGFR mediated rapid ligand degradation, which was completely inhibited by an inhibitor of lysosomal hydrolases, both mutant receptors mediated significantly slower rates of EGF degradation (Figure 3B). In concordance with the internalization assay, Y1045F was slightly more effective than the kinase-dead mutant, but even the latter mediated ligand destruction, probably because it can enter the basal route of endocytosis (Wiley *et al.*, 1991). In agreement with previous reports that documented a

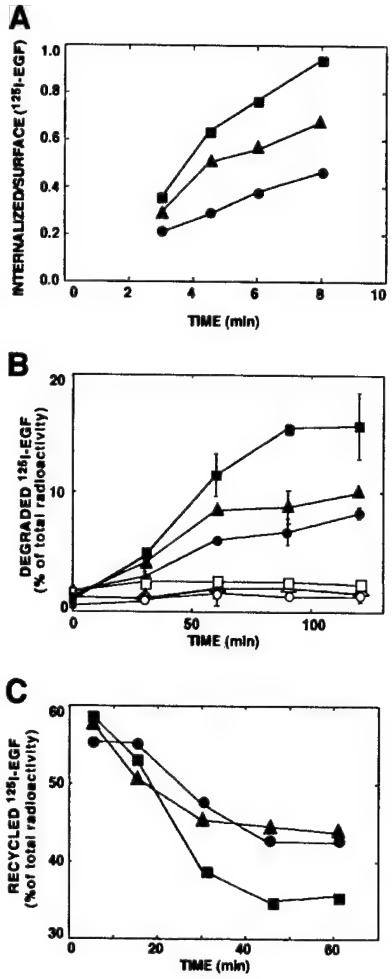

Fig. 3. The c-Cbl docking site of EGFR is essential for rapid ligand endocytosis and degradation, and it enables sequestration from the recycling pathway. (**A**) CHO cells transiently expressing a wt-EGFR (squares), a kinase-defective mutant receptor (circles) and a Tyr1045 mutant (Y1045F; triangles) were incubated at 37°C with a radiolabeled EGF (2 ng/ml). At the indicated times, cell monolayers were acid-washed to remove surface-bound EGF. Radioactivity present in the acidic fraction was quantified in triplicates and designated surface-associated ligand. The remaining cell-associated radioactivity (internalized) was similarly quantified following cell solubilization. The ratio obtained at each time point is presented (average ± SD). (**B**) Monolayers of CHO cells transfected as in (A) were incubated for 1 h at 20°C with a radiolabeled EGF. Sister monolayers were pre-incubated for 30 min with chloroquine (0.2 mM; open symbols). Thereafter, cells were washed and maintained at 37°C for the indicated time intervals. Media were then collected, and the cells were solubilized. The trichloroacetic acid-soluble radioactivity was determined and the fraction of degraded ligand presented. (**C**) CHO cells transiently expressing wt-EGFR (squares), the Y1045F mutant receptor (circles) or a kinase-defective mutant (triangles) were allowed to internalize a radiolabeled EGF (1 ng/ml) for the indicated time periods. The remaining surface bound ligand was removed by mild acid-washing. The EGF-loaded cells were then incubated with an unlabeled EGF at 4°C, followed by 1 h at 37°C. Intact radioactive ligand was harvested from the medium following a 1 h chase period. Similarly, the fraction of surface-bound radioactivity was determined and the combined fractions designated recycled ligand. The fraction of recycled ligand is presented as average ± SD (bars) of triplicate determinations.

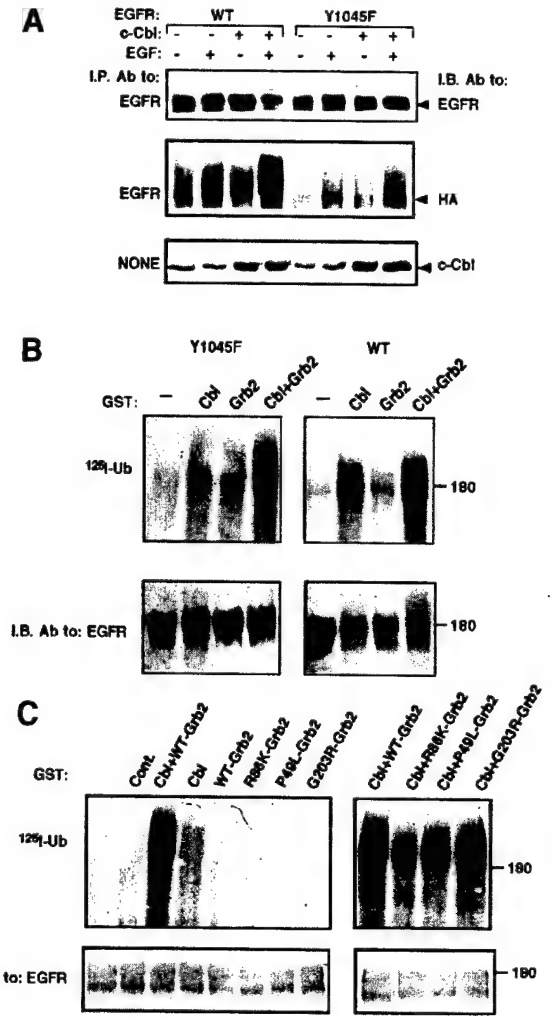

Fig. 4. An alternative receptor ubiquitylation pathway independent of Tyr1045 may involve Grb2. (**A**) CHO cells were transfected with plasmids encoding a wt-EGFR (WT) or the Y1045F mutant, along with vectors encoding c-Cbl and an HA-tagged ubiquitin. Following 48 h, cell monolayers were treated for 10 min at 37°C without or with EGF (100 ng/ml) and cell lysates subjected to immunoprecipitation (I.P.) and immunoblotting (I.B.) with the indicated antibodies. Whole-cell lysates were also analyzed (lower panel). (**B**) wt-EGFR or the Y1045F mutant receptor were immunopurified from transfected HEK-293 cells. Receptor immunoprecipitates were subjected to an *in vitro* ubiquitylation assay with a radiolabeled ubiquitin, c-Cbl, Grb2 or a combination of the two proteins was added to the reaction mixtures in the form of GST fusion proteins. Receptor immunoprecipitates were resolved by electrophoresis and proteins transferred to filters, which were first autoradiographed (upper panel, ^{125}I-Ub) and then immunoblotted with anti-EGFR antibodies (lower panel). (**C**) Immunoprecipitates of Y1045F were subjected to an *in vitro* ubiquitylation assay in the presence of one or two of the indicated GST fusion proteins. As a control, GST was added alone (Cont.) or it was omitted from the reaction (unlabeled lane).

defect in translocation of a kinase-defective EGFR to late endosomal compartments (Felder *et al.*, 1990; Hopkins *et al.*, 1990), this mutant recycled EGF more efficiently (Figure 3C). Interestingly, Y1045F was as effective as the kinase-dead receptor in recycling an intact ligand. In conclusion, Tyr1045-mediated interaction of EGFR with c-Cbl seems essential for rapid endocytosis of ligand-receptor complexes and their translocation to lysosomal sites of EGF degradation.

Grb2 enhances a surrogate mechanism of c-Cbl-dependent receptor ubiquitylation

Several results we obtained with the Y1045F mutant receptor raised the possibility that ligand-induced

endocytosis of the mutant receptor is only partially impaired. Assuming that c-Cbl and receptor ubiquitylation are essential for the residual ligand-dependent endocytosis of Y1045F, we examined the status of ubiquitylation of this mutant receptor upon high-level ectopic expression of c-Cbl. The results depicted in Figure 4A confirmed the ability of an overexpressed c-Cbl to enhance ligand-induced ubiquitylation and degradation of wt-EGFR. Likewise, faint but reproducible ubiquitylation of Y1045F was detectable upon short stimulation with EGF, and this modification was further enhanced when c-Cbl was overexpressed. In line with weak ligand-induced ubiquitylation, Y1045F underwent undetectable degradation following stimulation with EGF (Figure 4A). These results suggest the existence of a secondary mechanism of c-Cbl-induced receptor ubiquitylation. Because the surrogate pathway is sensitive to EGF but independent of Tyr1045, it seemed likely that another tyrosine phosphorylation site of EGFR is engaged.

The above results led us to suspect that a c-Cbl-associated protein, which is capable of binding a phosphotyrosine distinct from Tyr1045, is involved in recruiting c-Cbl to Y1045F. Because two autophosphorylation sites of EGFR can bind Grb2 (Batzer *et al.*, 1994; Okutani *et al.*, 1994), and this adaptor is constitutively and stoichiometrically bound to c-Cbl (Meisner and Czech, 1995; Donovan *et al.*, 1996), we reasoned that Grb2 may be involved in the surrogate pathway of EGFR ubiquitylation. To test this prediction, we utilized a previously described *in vitro* ubiquitylation assay (Levkowitz *et al.*, 1999; Waterman *et al.*, 1999). Figure 4B shows that incubation of an immuno-affinity purified wt-EGFR with reticulocyte lysate in the presence of a radiolabeled ubiquitin resulted in faint receptor ubiquitylation. However, addition of c-Cbl strongly promoted receptor ubiquitylation, as has been reported previously (Joazeiro *et al.*, 1999; Levkowitz *et al.*, 1999; Waterman *et al.*, 1999; Yokouchi *et al.*, 1999). In contrast, a recombinant Grb2 protein was ineffective, but its combination with c-Cbl moderately enhanced receptor ubiquitylation. This synergistic effect of Grb2 and c-Cbl was more conspicuous when the Y1045F mutant receptor was used *in vitro* as a substrate (Figure 4B). To test which domains of Grb2 are involved in Y1045F ubiquitylation, we used proteins carrying partially inactivating point mutations at each of the three domains of Grb2. Of the three mutants we tested, a protein mutated at the single SH2 domain (mutant denoted R86K-Grb2) was severely impaired in its ability to ubiquitylate Y1045F (Figure 4C), in line with binding to a phosphotyrosine of EGFR. On the other hand, a Grb2 protein mutated at the C-terminal SH3 domain (G203R-Grb2) was almost as active as wild-type Grb2, but a mutation within the N-terminal SH3 domain (mutant denoted P49L-Grb2) partly inactivated Grb2. Taken together, these results support recruitment of c-Cbl to Y1045F by simultaneous binding of Grb2 to c-Cbl (primarily via the N-terminal SH3 domain) and EGFR (via the SH2 domain).

The synergistic *in vitro* effect of Grb2 and c-Cbl prompted us to examine their combined action on EGFR in living cells. Overexpression of c-Cbl exerted only a moderate effect on ubiquitylation of the wt-EGFR (Figure 5A). However, co-expression of Grb2 and c-Cbl

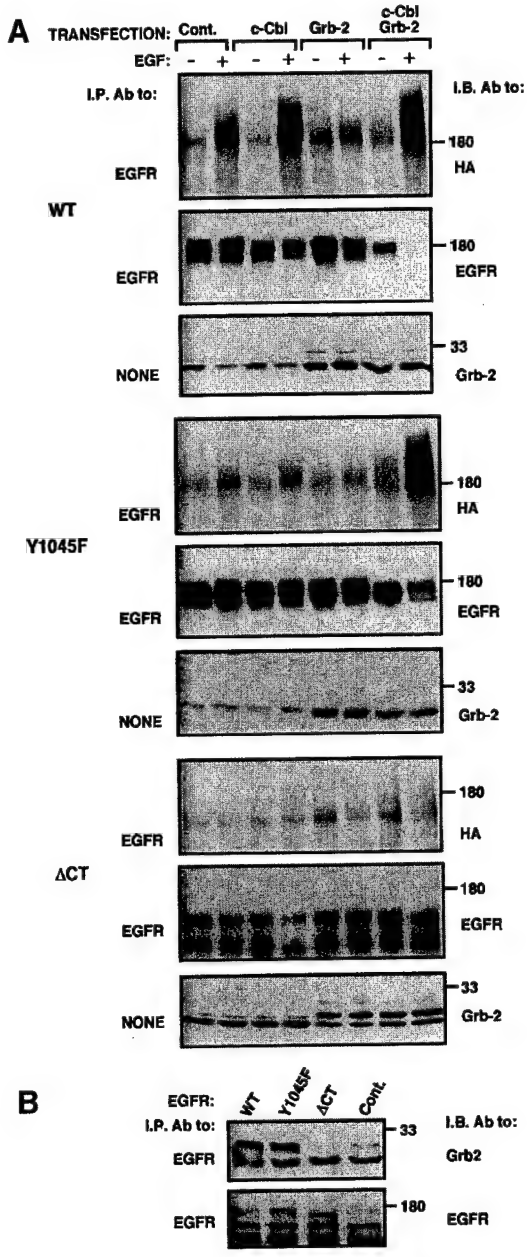

Fig. 5. Grb2 enhances c-Cbl-dependent ubiquitylation and degradation of EGFR in living cells. (**A**) Monolayers of CHO cells were transiently transfected with expression vectors encoding the indicated forms of EGFR. Alongside, plasmids encoding c-Cbl, Grb2, HA-ubiquitin and control empty vectors were used as indicated. Cell monolayers were treated for 10 min at 37°C without or with EGF (100 ng/ml). Subsequently, cell lysates were directly subjected to immunoblotting (I.B.; panels labeled NONE). Alternatively, EGFR was first isolated, and the immunoprecipitates analyzed with the indicated antibodies. (**B**) CHO cells were transfected with plasmids encoding the indicated forms of EGFR, along with plasmids encoding Grb2 and c-Cbl. A control culture was treated with an empty expression vector. Following stimulation with EGF (100 ng/ml; 10 min at 37°C), EGFR was isolated from cell lysates and the immuno-complexes analyzed by using antibodies to either Grb2 or EGFR.

enhanced receptor ubiquitylation and significantly increased its degradation. Moreover, when singly overexpressed, neither c-Cbl nor Grb2 could strongly enhance EGF-dependent ubiquitylation and degradation of Y1045F, but their combination effectively enhanced both

activities (Figure 5A). Because the effect of Grb2 was especially strong when cells were stimulated with EGF, we assumed that at least one of the two Grb2 association sites of EGFR [tyrosines 1068 and 1086 (Batzer *et al.*, 1994; Okutani *et al.*, 1994)] is involved in recruiting a complex of Grb2 and c-Cbl. This possibility was indirectly supported by the inability of a combination of Grb2 and c-Cbl to reconstitute ligand-induced ubiquitylation of a receptor lacking the whole C-terminus (ΔCT, residues 1–972), including all Grb2 and Shc association sites (Figure 5A). In line with loss of ligand-induced ubiquitylation, we observed no co-immunoprecipitation of the tail-less mutant receptor with Grb2 (Figure 5B), but both Y1045F and the wt-EGFR displayed physical association with the adaptor. In other experiments we analyzed EGFR mutants whose seven (mutant denoted F7) or eight (F8) potential phosphorylation sites, including Tyr1045, were replaced by phenylalanines. Whereas F8 lost ligand-induced ubiquitylation, F7 displayed some EGF-induced modification (data not shown), probably because its Tyr1114, a known Grb2 and Shc docking site, was intact.

Grb2 and c-Cbl co-operatively accelerate down-regulation and endocytosis of a mutant EGFR

To substantiate the combined effect of Grb2 and c-Cbl, we examined receptor down-regulation and endocytosis in CHO cells ectopically expressing the combination. As is evident from Figure 6A, overexpression of Grb2 alone exerted no significant effect on the extent of ligand-induced down-regulation of the wt-EGFR. This contrasts with c-Cbl, whose overexpression enhanced receptor down-regulation, in line with previous reports (Levkowitz *et al.*, 1998; Yokouchi *et al.*, 1999; Lill *et al.*, 2000). However, co-transfection of Grb2 and c-Cbl slightly enhanced receptor down-regulation (Figure 6A). The synergistic effect was more significant in the case of Y1045F, as this mutant underwent almost no ligand-induced down-regulation upon overexpression of either c-Cbl or Grb2 (Figure 6A). A complementary short-term ligand internalization assay reflected the lower rate of Y1045F internalization relative to wt-EGFR (Figures 3A and 6B). As noted previously (Levkowitz *et al.*, 1998; Thien *et al.*, 2001), we observed no significant effect of an overexpressed c-Cbl on the rate of internalization of the wt-EGFR. Likewise, Grb2 exerted no effect on internalization of either receptor form. However, the combination of Grb2 and c-Cbl reproducibly accelerated the rate of Y1045F internalization (Figure 6B). Taken together, these results extend the combined effect of c-Cbl and Grb2 to receptor endocytosis, implying causative relationships between receptor ubiquitylation and endocytosis.

The ability of Grb2 to enhance ligand-induced internalization of Y1045F was supported by fluorescence microscopy. As is shown in Figure 2, EGF and c-Cbl exerted only a small effect on endocytosis of

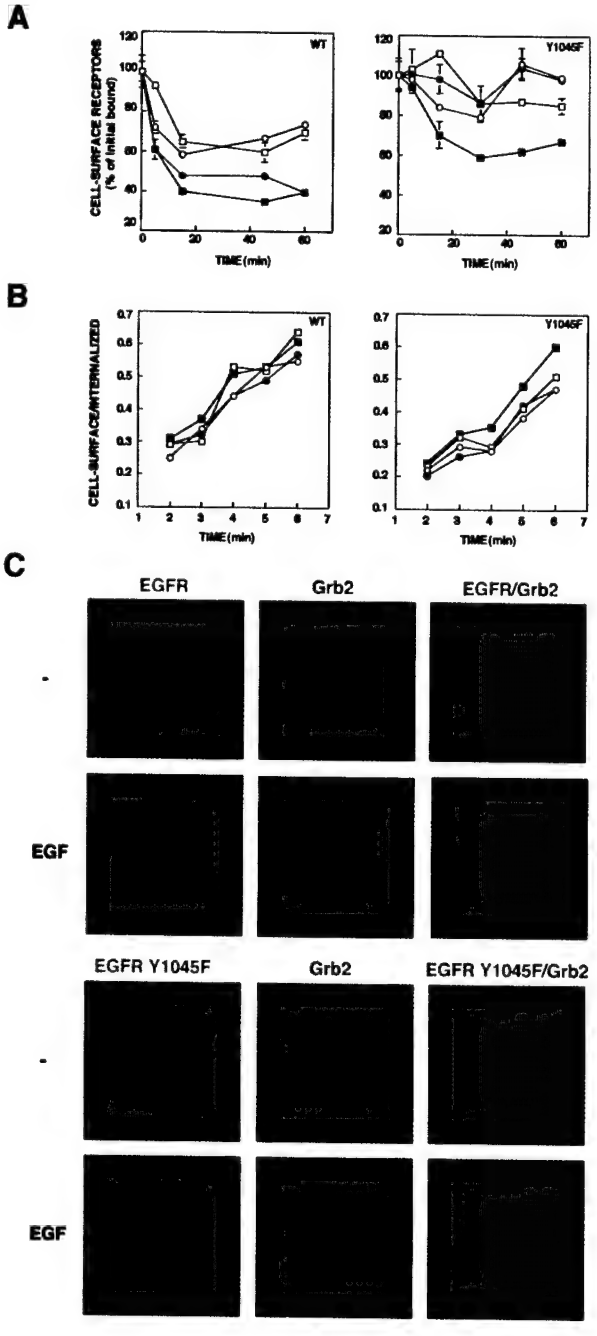

Fig. 6. Grb2 accelerates internalization and down-regulation of the Y1045F mutant EGFR. (**A**) CHO cells were transfected with expression vectors encoding wt-EGFR (WT) or the Y1045F mutant. Alongside we used plasmids encoding c-Cbl (closed circles), Grb-2 (open squares), a combination of Grb2 and c-Cbl (closed squares) or an empty control vector (open circles). Forty-eight hours post-transfection, cultures were incubated at 37°C with EGF (25 ng/ml) for the indicated periods of time. Cell-bound ligand was removed, and the level of surface receptors was determined by binding of a radiolabeled EGF at 4°C. The average ± SD (bars) of triplicate determinations is shown for each time point. (**B**) Monolayers of CHO cells were transfected as in (A) with vectors driving expression of the indicated version of EGFR. After 48 h, cells were incubated at 37°C with a radiolabeled EGF (2 ng/ml). At the indicated time points, monolayers were acid-washed to remove surface-bound ligand. Radioactivity present in the acidic fraction was designated surface-associated ligand. The remaining cell-associated radioactivity was determined in triplicates following cell solubilization and designated internalized ligand. Symbols are as in (A). (**C**) CHO cells transiently overexpressing HA-tagged c-Cbl and histidine-tagged Grb2, along with a GFP–EGFR, or a fusion protein containing a mutation at Tyr1045 (Y1045F), were grown on cover slips for 48 h after transfection. Thereafter, cells were incubated for 15 min at 37°C without or with EGF (100 ng/ml). To visualize Grb2, cells were fixed, permeabilized, and incubated with a rabbit anti-His_6 followed by a Cy3-conjugated secondary antibody (red). The GFP–EGFR fluorescence is shown in the left column (green). The right column presents the overlay of GFP and Cy3 fluorescence; a yellow color is seen in areas of co-localization.

GFP–Y1045F. However, expression of an ectopic Grb2 together with c-Cbl enhanced ligand-induced endocytosis of GFP–Y1045F (Figure 6C). Interestingly, we observed partial co-localization of Grb2 with the internalized EGFR (note the yellow figures in the merge panels of Figure 6C). Indeed, a recent report utilizing fluorescence energy transfer detected physical association between Grb2 and EGFR in endosomes (Sorkin *et al.*, 2000). Together with the biochemical lines of evidence, our morphological analyses support the existence of a dual mechanism of c-Cbl-induced endocytosis: a major pathway mediated by Tyr1045 and c-Cbl, and a second route that involves recruitment of Grb2, presumably pre-complexed with c-Cbl.

The RING finger and the regulatory region of c-Cbl, but not the SH2 domain, are required for Grb2-mediated receptor ubiquitylation

We next analyzed the domains of c-Cbl that are necessary for interaction with Grb2 and for the associated increase in Y1045F ubiquitylation. Three mutants of c-Cbl were used: a protein whose SH2 domain is defective due to a point mutation [G306E (Bonita *et al.*, 1997)] and two deletion mutants lacking either the N-terminal or the C-terminal half [Cbl-C and Cbl-N(RF), respectively]. Both deletion mutants carried an intact RING finger, but only Cbl-N(RF), whose SH2 domain is intact, could support ubiquitylation of wt-EGFR (data not shown; Lill *et al.*, 2000). In contrast, this mutant displayed only weak co-immunoprecipitation with Grb2 (Figure 7A), and when co-expressed together with the adaptor it mediated no increase in ubiquitylation and degradation of Y1045F (Figure 7C). Thus, recruitment of Grb2 to the C-terminus of c-Cbl appears to be essential for the surrogate pathway of EGFR ubiquitylation, but the presence of the SH2 domain is not critical. Indeed, a mutant containing an intact poly-proline domain, the site of Grb2 binding (Meisner and Czech, 1995; Donovan *et al.*, 1996), but lacking the SH2 domain (Cbl-C), retained binding to Grb2 (Figure 7A) and partially enhanced Y1045F ubiquitylation and degradation (Figure 7C). In experiments that are not presented, we examined the relative ability of native c-Cbl

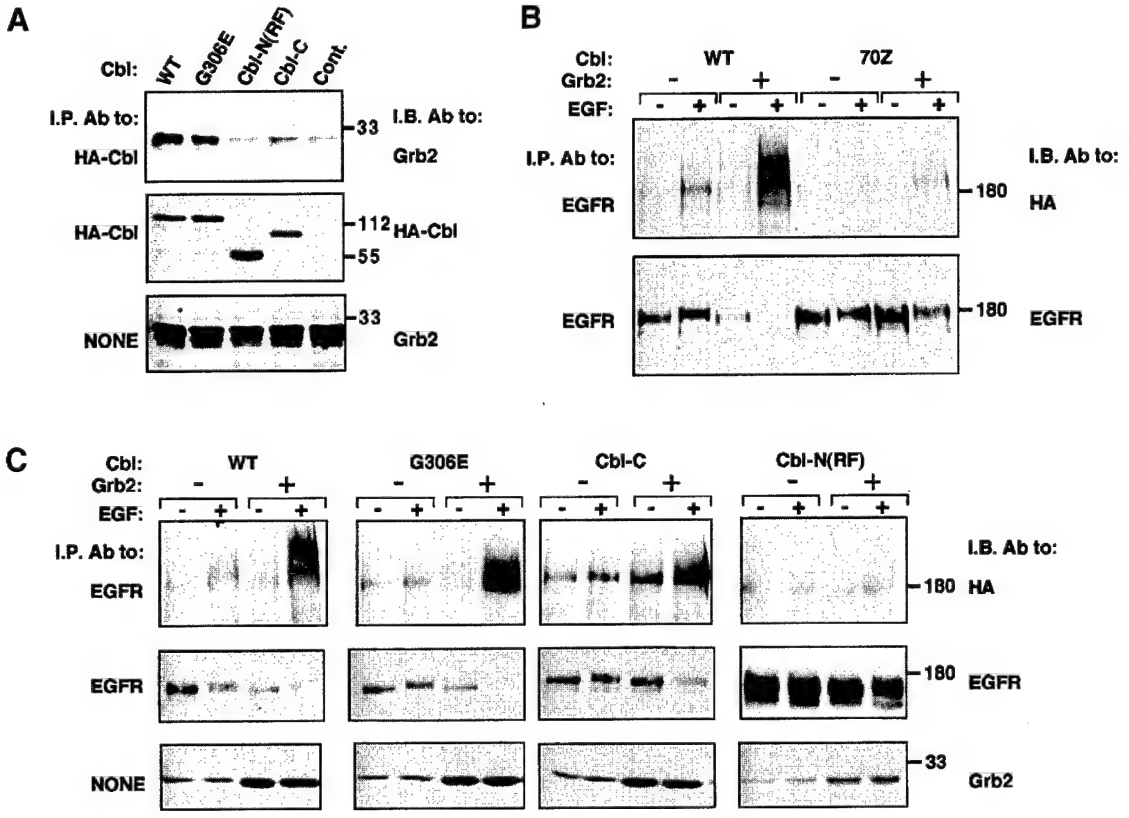

Fig. 7. Subdomains of c-Cbl involved in receptor ubiquitylation. (**A**) CHO cells were transfected with plasmids encoding EGFR, Grb2, the indicated HA peptide-tagged mutants of c-Cbl or a control empty vector. Whole-cell extracts were analyzed by immunoblotting either directly or after immunoprecipitation of c-Cbl. The following mutants of c-Cbl were used: a protein the SH2 domain of which is defective (G306E); and deletion mutants containing either the N-terminal half [Cbl-N(RF), residues 1–429] or the C-terminal half (Cbl-C, residues 338 to end). (**B**) Monolayers of CHO cells were transiently transfected with the Y1045F mutant of EGFR, along with wild type (WT) or the 70Z mutant of c-Cbl, in the presence or absence of a Grb2 expression vector. Receptor ubiquitylation was detected using a HA-tagged ubiquitin expression vector. Forty-eight hours post-transfection, monolayers were incubated without or with EGF (100 ng/ml). Cell lysates were subjected to immunoprecipitation (I.P.) with an anti-EGFR antibody and immunoblotting (I.B.) with either an antibody to HA-ubiquitin or an antibody recognizing EGFR. (**C**) CHO cells were co-transfected with vectors driving expression of the indicated c-Cbl mutants [see (A)], along with a plasmid encoding the Y1045F mutant receptor. As indicated, transfections were performed with a plasmid encoding Grb2 or a control empty vector. All transfections were carried out in the presence of a plasmid directing expression of a HA-tagged ubiquitin. Cells were treated for 10 min at 37°C without or with EGF (100 ng/ml). Whole-cell lysates were analyzed by immunoblotting (I.B.) either directly (panels labeled NONE) or following immunoprecipitation (I.P.).

and Cbl-N(RF) to enhance ubiquitylation and degradation of wt-EGFR. As expected, the mutant was inferior to c-Cbl, probably due to evading the surrogate pathway. By analyzing a natural mutant of the RING finger, namely 70Z-Cbl, we learned that this domain is essential for the surrogate pathway of receptor ubiquitylation (Figure 7B). In contrast, by using the G306E mutant, we confirmed that the SH2 domain of c-Cbl is not involved in the surrogate pathway. In conclusion, unlike the major pathway of receptor ubiquitylation, which requires binding of the SH2 domain of c-Cbl to a specific phosphotyrosine of the receptor, the surrogate pathway is independent of the c-Cbl's SH2 domain. In addition to the RING finger, this pathway involves the C-terminal half of c-Cbl, probably because this portion of the molecule contains several proline-rich sites that recruit Grb2.

Discussion

Receptor endocytosis terminates signal transduction

Several previous attempts to block EGFR internalization, by either receptor mutagenesis (Wells *et al.*, 1990) or a mutant of dynamin (Vieira *et al.*, 1996), concluded that endocytosis terminates the mitogenic activity of EGF. Nevertheless, the effect of receptor endocytosis on signaling potency remained a matter of some controversy (Di Guglielmo *et al.*, 1994; Emlet *et al.*, 1997; Haugh *et al.*, 1999). In part, this issue is complicated by the existence of at least two routes of receptor endocytosis: the ligand-activated route requires the intrinsic tyrosine kinase activity of EGFR. In contrast, the slower pathway is constitutive, and unlike the inducible pathway it primarily recycles receptors back to the cell surface (Wiley *et al.*, 1991). By resolving the role of c-Cbl in receptor endocytosis and sorting to destruction, our results favor the notion that receptor endocytosis terminates mitogenic signaling. Thus, a c-Cbl's site mutant receptor (Y1045F) with defective endocytosis (Figures 2 and 3A) and increased recycling (Figure 3C) is endowed with enhanced signaling capacity (Figure 1).

Previous attempts to construct an internalization-defective mutant of EGFR led to the realization that the 213 C-terminal amino acids of EGFR include an inhibitory domain and an internalization region (amino acids 973–1022). It is interesting to note that Tyr1045 is located outside of the mapped internalization region. However, consistent with its role in endocytosis, genetic analyses of vulva formation in *C.elegans* attributed an inhibitory role to the respective residue (Yoon *et al.*, 2000). In addition, mutagenesis of EGFR has identified residues 1022–1123 as a region required for lysosomal degradation (Kornilova *et al.*, 1996). Consistent with the association of internalization with decreased mitogenic signaling, virulence of several strains of the oncogenic avian erythroblastosis virus, which encodes an ortholog of EGFR, share deletions of a region encompassing the c-Cbl-specific docking site (Shu *et al.*, 1991). Likewise, this site is missing in oncogenic forms of the human EGFR, which are frequently found in brain tumors (Ekstrand *et al.*, 1992). In a complementary way, the transforming activity of some ubiquitylation-defective forms of c-Cbl depends on binding to EGFR (Thien *et al.*, 2001), and targeted inactivation of c-Cbl sensitizes macrophages to the colony stimulating factor-1 (Lee *et al.*, 1999).

A linkage between receptor ubiquitylation and endocytosis

Previous attempts to resolve the relationships between receptor ubiquitylation and endocytosis reported no effect of an overexpressed c-Cbl on the rate of EGFR endocytosis (Levkowitz *et al.*, 1998; Thien *et al.*, 2001). This observation and the ligand-induced co-translocation of c-Cbl and EGFR to endosomes led us to suggest that the effect of c-Cbl on receptor sorting occurs at the early endosome (Levkowitz *et al.*, 1998). By using a receptor mutant incapable of c-Cbl recruitment (Y1045F), we identified an earlier effect of c-Cbl. Apparently, ubiquitylation by c-Cbl occurs at the plasma membrane or very close to it, and it determines the rate of receptor endocytosis. It is conceivable that c-Cbl-mediated ubiquitylation of a cell surface-localized EGFR dictates sorting to the invaginating clathrin-coated pit. This conclusion is consistent with the ability of a mutant dynamin to block endocytosis, but not ubiquitylation of EGFR (Stang *et al.*, 2000).

Collectively, the observations made with an overexpressed c-Cbl and the Y1045F mutant receptor lead us to conclude that sorting of internalized EGFRs is a progressive process. It probably initiates at the cell surface upon limited c-Cbl-mediated ubiquitylation, which accelerates endocytosis (de Melker *et al.*, 2001). However, ubiquitylation seems to proceed *en route* to the late endosome/pre-lysosome, because c-Cbl and EGFR remain physically associated (Levkowitz *et al.*, 1998), and blocking receptor internalization prevents maximal ubiquitylation (Stang *et al.*, 2000). The emerging model is thus reminiscent of the mechanism regulating endocytosis in yeast (reviewed in Hicke, 2001). Mono-ubiquitylation seems sufficient to signal internalization of several yeast proteins into primary endocytic vesicles. Apparently, ubiquitin itself carries an internalization signal, because fusion of a lysine-less ubiquitin to several yeast and mammalian proteins enhances their rate of internalization (Nakatsu *et al.*, 2000 and references therein). It is therefore possible that c-Cbl enhances the rate of EGFR endocytosis by appending an internalization signal intrinsic to ubiquitin.

The dual role of Grb2

The Grb2 adaptor protein is involved in many signal transduction pathways, including those initiated by growth factors, antigens and antibodies. Its single SH2 domain allows inducible binding to tyrosine-phosphorylated proteins, whereas the two flanking SH3 domains recruit signaling proteins, including the guanine nucleotide exchange protein Sos and the E3 ubiquitin ligase, c-Cbl (Fukazawa *et al.*, 1995). The interaction with Sos is relatively well characterized; it recruits the exchange protein to the plasma membrane and activates the Ras pathway. However, although a complex of c-Cbl and Grb2 is abundant in many cell types and its dissociation may be regulated (Buday *et al.*, 1996; Donovan *et al.*, 1996), the functional consequences of recruiting a c-Cbl–Grb2 complex to activated receptors is currently unknown. The results presented in this study indicate that one function of

Grb2–Cbl interaction is to negatively regulate signaling by mediating receptor degradation. Interestingly, genetic evidence raised the possibility that Grb2 recruits another negative regulator of EGFR in worms, namely Ark-1 (Hopper *et al.*, 2000). However, unlike c-Cbl/Sli-1, the mechanism underlying the Ark-1 pathway of receptor desensitization remains unknown.

Although our conclusions rely on overexpression, and therefore they must be confirmed by additional approaches, it is conceivable that two distinct mechanisms underlie inducible degradation of EGFR. The major one is mediated by Tyr1045, which directly binds to the SH2 domain of c-Cbl. The secondary mechanism seems to involve one of the Grb2-binding sites of EGFR, and indirect recruitment of c-Cbl through its constitutive interactions with an SH3 domain of the adaptor. The relative contribution of the two pathways to EGFR desensitization will require further investigation; according to a previous report a c-Cbl mutant incapable of Grb2 binding is as effective as the wild-type protein (Lill *et al.*, 2000), but the data we presented and additional preliminary results suggest that in some cell lines, coupling to Grb2 may contribute to the overall extent of receptor degradation.

Additional open questions relate to the possible involvement of the many other c-Cbl-associated proteins, like the adaptors Nck, Shc and Crk. The involvement of Grb2, however, was predictable because of two lines of evidence. First, microinjection of a recombinant SH2 domain of Grb2 inhibited endocytosis of EGFR (Wang and Moran, 1996). Secondly, deletion of the Grb2-binding poly-proline motif of SLI-1 significantly affected vulva formation in transgenic worms (Yoon *et al.*, 2000). Together with the results we have presented, these lines of evidence attribute to Grb2 a dual role in signaling; along with the extensively characterized stimulatory activity of the Ras pathway through binding to SOS, the adaptor also initiates the process of receptor degradation by recruiting c-Cbl. Because c-Cbl and SOS do not co-immunoprecipitate (Meisner *et al.*, 1995; Fukazawa *et al.*, 1996), they seem to form exclusive complexes with Grb2. Thus, it is conceivable that by alternative interaction with SOS and c-Cbl, Grb2 integrates both positive and negative inputs to signaling pathways.

In summary, c-Cbl critically controls receptor fate: its close apposition to and phosphorylation by EGFR initiate receptor ubiquitylation. Apparently, ubiquitylation of EGFR identifies it for rapid endocytosis, which starts the process of homologous desensitization. However, c-Cbl remains associated with the receptor throughout the endocytic compartment, probably in order to allow effective sorting to degradation. Evidently, several mechanisms of c-Cbl recruitment exist in cells: the major route involves the SH2 domain of c-Cbl and autophosphorylation at Tyr1045 of EGFR, which is situated within a lysosome-targeting motif. In contrast, the surrogate route bypasses the lysosome-targeting motif. Apparently, c-Cbl is recruited to this route in a complex with the Grb2 adaptor, which engages with EGFR through several tyrosine autophosphorylation sites. Future studies will address functional characteristics of each route of receptor ubiquitylation, as well as the relationships to positive signaling through the adaptors Grb2 and Shc.

Materials and methods

Materials

Rabbit antibodies to c-Cbl (C-15), a His_6 tag and EGFR were from Santa Cruz Biotechnology (Santa Cruz, CA). A monoclonal antibody (mAb) to Grb2 was from Transduction Laboratories (Lexington, KY) and an anti-hemagglutinin (HA) mAb was purchased from Boehringer Mannheim. A mAb to the active doubly phosphorylated form of Erk was from Sigma. The composition of buffers was as described previously (Waterman *et al.*, 1999).

Plasmid construction and transfection of expression vectors

Mammalian expression plasmids for wild-type and mutant EGFR, c-Cbl, glutathione *S*-transferase (GST)–Cbl and Cbl-C have been described previously (Levkowitz *et al.*, 1999). The Cbl-N(RF) deletion mutant was prepared by introducing a stop codon next to the codon encoding amino acid 429. GST–Grb2 mutants were obtained from Dr Jan Sap (New York University). A His_6 tag was inserted at the C-terminus of the human Grb2 cDNA by PCR amplification. Expression vectors were introduced to CHO cells by using the LipofectAMINE transfection method (Gibco-BRL). The total amount of DNA in each transfection was normalized with the pCDNA3 plasmid.

Cell proliferation and SRE transcription assays

Cells were washed free of IL-3, resuspended in RPMI 1640 medium at 5×10^5 cells/ml, and treated without or with growth factors or IL-3. Cell survival was determined by using the MTT assay (Mosman, 1983). The SRE transcription assay was performed as described previously (Waterman *et al.*, 1999).

Lysate preparation, immunoprecipitation and western blotting

Following stimulation, cells were extracted in solubilization buffer, and lysates cleared by centrifugation. The proteins in the lysate supernatants were immunoprecipitated for 2 h at 4°C. The immunoprecipitates were washed three times with HNTG buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 0.1% Triton X-100 and 10% glycerol), resolved by electrophoresis and electrophoretically transferred to a nitrocellulose membrane. Membranes were blocked for 1 h in Tris–HCl-buffered saline containing 1% milk, blotted for 2 h with a primary antibody (1 μg/ml), followed by a secondary antibody (0.5 μg/ml) linked to horseradish peroxidase. Immunoreactive protein bands were detected using the enhanced chemiluminescence reagent (Pharmacia-Amersham).

Biochemical analyses of endocytosis

Receptor down-regulation was performed as described previously (Levkowitz *et al.*, 1998). To follow receptor recycling and to monitor EGF internalization, we used the previously described protocols of Kornilova *et al.* (1996) and Sorkin *et al.* (1993), respectively. Ligand degradation was assayed as follows: conditioned media containing radiolabeled ligands were assayed by the addition of cold trichloroacetic acid (TCA) (10% final concentration). Samples were precipitated after 1 h at 2°C by centrifugation. Radioactivity present in the supernatants and pellets (TCA-insoluble fraction) was determined.

In vitro assay for receptor ubiquitylation

Receptors were immunoprecipitated from cleared lysates of transfected HEK-293T cells. Following isolation, agarose beads were extensively washed with HNTG followed by an additional wash with ubiquitin wash buffer (5 mM $MgCl_2$, 40 mM Tris–HCl pH 7.5). Agarose beads were then resuspended in buffer containing 40 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM dithiothreitol, 2 mM ATP-γ-S and 3 μg/ml radiolabeled ubiquitin supplemented with crude rabbit reticulocyte lysate (5 μl; Promega) and the indicated recombinant proteins (5 μg), and incubated for 1 h at 30°C. The beads were then washed extensively and EGFR eluted with gel sample buffer.

Immunofluorescence

Transfected cells grown on cover slips were incubated for 15 min at 37°C with or without EGF (100 ng/ml). The cells were then fixed for 15 min with 3% paraformaldehyde in phosphate-buffered saline (PBS). Fixed cells were washed, and permeabilized for 10 min at 22°C with PBS containing 1% albumin and 0.2% Triton X-100. For labeling, cover slips were incubated for 1 h at room temperature with an anti-HA antibody or a rabbit antibody to His_6 peptide tag. After extensive washing in PBS, the cover slips were incubated for an additional 1 h with a Cy3-conjugated

secondary antibody. The cover slips were mounted in mowiol. Confocal microscopy was performed using a Zeiss Axiovert 100 TV microscope (Oberkochen, Germany) with a 63X/1.4 plan-Apochromat objective, attached to the Bio-Rad Radiance 2000 laser scanning system, operated by LaserSharp software. Figures were taken from middle sections of cells.

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The role of ubiquitylation in signaling by growth factors: implications to cancer

Keren Shtiegman, Yosef Yarden*

Department of Biological Regulation, The Weizmann Institute of Science, Rehovot 76100, Israel

Abstract

Cancer cells depend on multiple, locally produced growth factors. Signaling by growth factors entails phosphorylation events, and its termination is determined primarily by endocytosis of growth factor receptor complexes. One group of growth factor receptors frequently implicated in human cancer is the ErbB family of receptor tyrosine kinases. By using ErbB as a prototype, here we review the role of protein ubiquitylation in the process that terminates signaling. Specifically, we concentrate on several adaptor proteins, including c-Cbl and Hgs, to elucidate the complexity of receptor sorting for degradation. Detailed understanding of ubiquitylation control on receptor desensitization may lead to better ways to diagnose and eradicate cancer.

Keywords: Endocytosis; Growth factor; Oncogene; Signal transduction; Ubiquitin

1. Introduction

The first indication that cancer can be attributed to natural causes was when Hippocrates incorrectly described cancer as an imbalance between the black humor (from the spleen) and the other three bodily humors: blood, phlegm, and bile. References to 'cancer houses,' 'cancer families,' and 'cancer villages' in the Middle Ages illustrate a then emerging concept that cancer might be an inherited or an environmental disease. Today we know that certain molecules are often mutated in cancers, and these include proteins governing cellular growth, differentiation and developmental signals. An example is the ErbB family—a prototype of the large family of receptor tyrosine kinases (RTKs; for a recent review, see [1]). The first evidence that ErbB family members play a role in cancer was obtained in the early 1980s when the avian erythroblastosis virus (AEV) was found to encode an aberrant form of one of the ErbB family members, namely the epidermal growth factor receptor (EGFR) [2]. Mutations, overexpression, structural rearrangements, disruption of autocrine/paracrine loops, and liberation from regulatory constraints are other means by which transforming viruses exploit the EGFR signaling pathway.

The ErbB family of transmembrane receptors consists of the EGFR (EGFR/ErbB-1), Neu/ErbB-2, ErbB-3/HER3 and ErbB-4/HER4. Irrespective of structural similarities within the ErbB family, ligand specificity, substrate selectivity, and potency of kinase activity differ dramatically. The most intriguing example of such diversification within the ErbB family is that to date ErbB-2 is an orphan receptor but contains an active kinase domain which profoundly affects signaling when activated by other ErbB members, while ErbB-3 is devoid of kinase activity [3], but nevertheless, this receptor binds numerous ligands. In similarity to other RTKs, the monomeric forms of the receptors are inactive. A number of different ligands activate the receptors by binding to the extracellular domain and consequently inducing the formation of receptor dimers [4]. In fact, all 10 homo- and heterodimers are formed and they display distinct selectivity that defines a hierarchical relationship [5,6]. Subsequent to dimerization, tyrosine residues on one receptor are presumably transphosphorylated by the other member of the receptor pair, thereby forming docking sites for phosphotyrosine-binding proteins, which in turn have the potential to induce a vast array of signal transduction cascades.

2. Mechanisms for the attenuation of ErbB signaling

Under normal cellular conditions, the ErbB family is involved, among other functions, in epithelial development, renewal and remodeling. Remarkably, because this signaling module serves as a major driving force for cell proliferation, it has the potential to be exploited in cellular transformation. It, therefore, comes as no surprise that the ErbB signaling

* Corresponding author. Tel.: +972-8-9343974; fax: +972-8-9342488.
E-mail address: yosef.yarden@weizmann.ac.il (Y. Yarden).

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network is tightly regulated. Important lessons relevant to signal attenuation have been learned by studying invertebrate systems. The EGFR signaling module is highly conserved throughout evolution. Although less complex, both the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster* have versions of the EGFR signaling pathway [7]. In *C. elegans*, the induction of the vulva depends on the activity of Let-23, the only EGFR homologue. Genetic analyses have indicated that sli-1/cbl [8], gap-1/RasGAP [9], and Ark-1/ACK [10] all keep the activity of Let-23 in check. In *D. melanogaster*, the EGFR homologue DER, is key to a number of developmental stages including oogenesis, embryogenesis, and wing and eye development. Examples of negative regulators of DER signaling comprise of a unique secreted ligand, Argos [11], a transmembrane protein called Kekkon 1 [12], and an intracellular protein, Sprouty [13].

As in the case of lower organisms, negative regulators target the ErbB signaling pathway at a number of junctures. For example, the formation of hetero-oligomers of active receptors with inactive alternatively spliced receptor mutants is expected to downmodulate signaling [14]. An additional mechanism is the inhibition of ErbB-induced mitogenic signaling by the receptor-associated late transducer (RALT) [15]. Furthermore, tyrosine-specific protein phosphatases may inhibit the ErbB signaling cascade at many points during signal propagation. The most prominent regulator of ErbB signal termination is downregulation—a term used to denote the desensitization of receptors by the removal of activated receptors from the cell surface by accelerated endocytosis. The value of such a mechanism can be inferred from the finding that a receptor that is endocytosis impaired will result in enhanced mitogenic signaling [16]. Interestingly, the kinetics and destiny of downregulated ErbB family members vary depending upon the type of ErbB homo- and heterodimers formed, and additionally the ligand that stimulated their formation [17,18].

3. Receptor endocytosis: an overview

The endocytic mechanism consists of a number of stages, and consequently cannot be solely considered as a signal termination mechanism. The vast array of cellular outcomes mediated by the ErbB signaling pathway reveals that it is not only the on/off mechanism that is important but the signaling kinetics and magnitude [19]. Emerging data indicate that the specificity, kinetics, and magnitude of the receptor response may be regulated by the localization of the activated receptor in the endocytic pathway [20]. Several functionally distinct pathways of endocytosis exist in cells, such as phagocytosis, pinocytosis, and receptor-mediated endocytosis. Because of its prevalence, we will concentrate on ligand-stimulated, receptor-mediated endocytosis, a clathrin-coated pit-dependent mechanism (see Fig. 1).

The capability of clathrin-coated vesicles (CCVs) to selectively sequester protein cargo into a membrane vesicle is dependent upon three major components. Firstly, the self-assembling property of the clathrin coat, which is contingent upon the triskelion shape of clathrin and its intrinsic ability to form a polyhedral lattice. Secondly, the well-established AP2 adaptor complex which is drawn into the lattice and triggers CCV formation at the plasma membrane, while incorporating transmembrane molecules by associating with the cytoplasmic domains of these proteins. Thirdly, dynamin, a GTPase responsible for fission of the vesicle from the plasma membrane resulting in the detachment of the CCV from the donor membrane (reviewed in [21]). In essence, the formation of the CCV may provide the initial cargo selection function. The coated vesicle then undergoes clathrin shedding and fuses with an acceptor compartment forming the early endosome, where the second major sorting event occurs. Although it remains disputed whether trafficking from the early endosome to the next compartment in the endocytic pathway involves vesicular transport or maturation, the cargo proteins proceed into a compartment referred to as either the late endosome or the multi-vesicular body (MVB). This compartment is characterized by different protein composition, low luminal pH, and multiple internal vesicles. Finally, the cargo that is not recycled back to the plasma membrane is degraded in the lysosome.

4. Two distinct clathrin-dependent pathways of endocytosis

Several observations led to the realization that clathrin-dependent endocytosis is a pathway utilized by two independent modes of endocytosis. Two receptors, namely transferrin receptor (TfR) and EGFR have different limiting steps at an early point in their endocytosis, although they may utilize the same coated pits [22]. Furthermore, unlike in the case of TfR, a mutation that blocks EGFR–AP2 interactions has no effect on the rate of receptor internalization [23]. Likewise, mutations in the μ-subunit of AP2 that block recognition of internalization motifs can abrogate TfR internalization, but they do not affect EGFR uptake [24]. In conclusion, the endocytic pathway may be sub-divided into constitutive endocytosis, which is dependent on short linear internalization motifs as in the case of TfR, and regulated endocytosis that relies on additional interactions as in the case of EGFR.

4.1. Constitutive endocytosis

The low density lipoprotein (LDL) and TfRs are internalized by virtue of their constitutive binding to AP2, and subsequent recruitment to clathrin-coated pits. In contrast to ligand-induced endocytosis, receptors undergoing constitutive endocytosis may not actively promote coat assembly, but they may engage into preformed coats. Natural mutations in the LDL receptor helped uncover the determinants required for constitutively internalizing receptors to bind the AP2

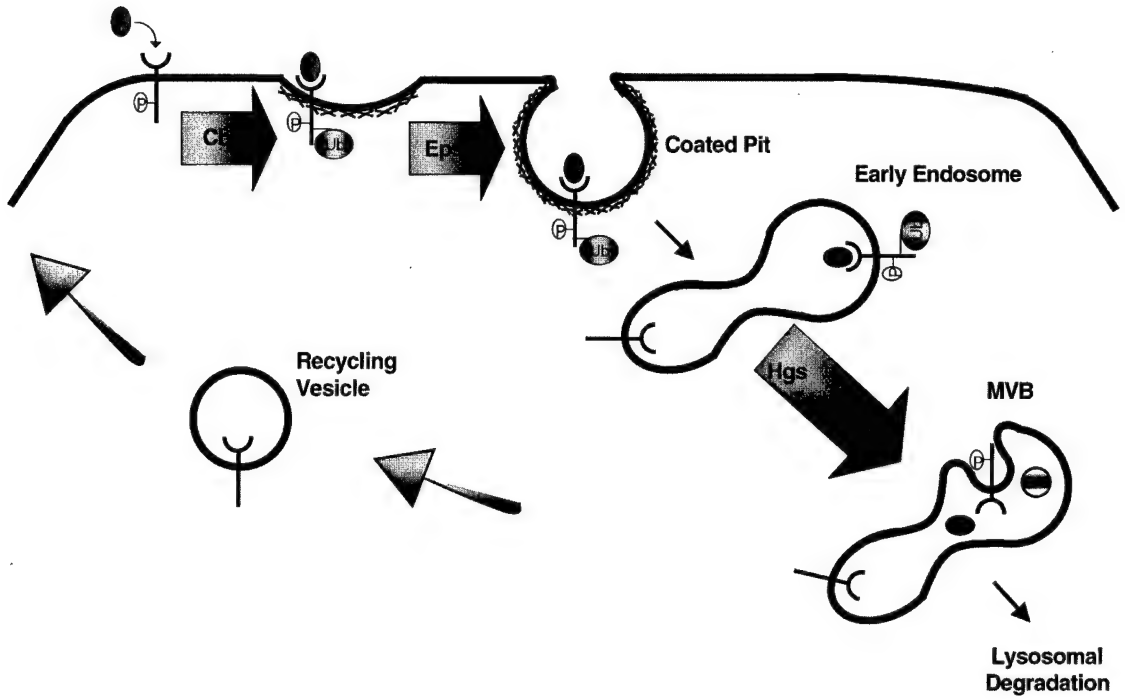

Fig. 1. Receptor-mediated endocytosis of EGF. Upon EGF binding and receptor auto-phosphorylation, c-Cbl is recruited to phosphorylated tyrosine 1045 of EGFR. Once in a complex with an active EGFR, c-Cbl undergoes tyrosine phosphorylation and promotes ubiquitylation of both the receptor and several receptor-associated signaling proteins. Apparently, ubiquitylated receptor molecules are recognized by a multi-protein complex that sorts them to an invaginating clathrin-coated pit. These 'loaded' clathrin-coated regions, with the regulation of a number of adaptors such as Eps15, engulf the activated receptor in a dynamin-dependent manner to form a clathrin-coated vesicle (not shown). The shedding of clathrin, concomitant with a reduction in internal pH and the accumulation of hydrolytic enzymes, give rise to the early endosome. Passage from the early to the late endosome consists of a major sorting event dependent upon c-Cbl and adaptor proteins such as Hgs and Tsg101. Proteins destined for degradation proceed to the lysosome. Recycling of receptors back to the cell surface may occur from most endosomal compartments, however, as the receptor proceeds along the endocytic pathway, the efficiency of recycling declines.

complex [25]; these determinants are internalization signals located within the cytoplasmic domains of transmembrane proteins. A variety of internalization motifs have been identified in mammalian cells and may be sub-categorized into two groups [26]. The first group is characterized by an essential tyrosine, such as the YXXØ motif (where X is any amino acid and Ø is an amino acid with a bulky hydrophobic group), or NPXY. The second class of internalization signals typically contains a dileucine sequence.

4.2. Regulated endocytosis

Regulated endocytosis may be further sub-divided into ligand-dependant, stress-induced, and antibody-mediated endocytosis. Upon activation, EGFR molecules cluster over clathrin-coated regions of the plasma membrane. These 'loaded' clathrin-coated regions invaginate in a dynamin-dependent manner to form endocytic vesicles. The endocytic vesicles then undergo maturation concomitant with a reduction in internal pH and the accumulation of hydrolytic enzymes. Continuous activation of tyrosine phosphorylation leads to the recruitment of c-Cbl [27], a ubiquitin ligase that directs activated RTKs to lysosomal degradation by tagging them with poly-ubiquitin chains [28–31].

The fundamental difference between constitutive and ligand-induced endocytosis does not only lie within the kinetics of endocytosis, nor in the delicate balance between recycling and degradation, but in the unquestionable requirement of an additional mechanism distinct from the classical internalization cargo-intrinsic signals. Contrary to initial models, ligand binding does not stimulate unmasking of internalization signals and recognition of the receptor by AP2 molecules. Instead, accumulating evidence suggests the possibility that EGFR's independence of AP2 may be due to phosphorylation events. By analyses of EGFR mutants, it was found that internalization motif-independent endocytosis of EGFR still requires an active tyrosine kinase domain [32]. Because addition of a soluble tyrosine kinase recovered endocytosis of a kinase-dead mutant receptor, these findings suggest that phosphorylation of a yet undefined factor, which may act as a 'connector', is required for efficient recruitment of EGFR into coated pits. One such factor may be the Src-dependent tyrosine phosphorylation of clathrin, abrogation of which inhibits both clathrin redistribution and EGFR endocytosis [33]. The recent emergence of a growing number of multivalent adaptor proteins involved in endocytosis (e.g. c-Cbl) and the findings that some of these are substrates of the EGFR-signaling pathway

indicate that more factors may act in concert to influence endocytosis of EGFR.

5. Major players in EGFR endocytosis—endocytic adaptors

The accessory proteins that have been shown to participate in some aspects of clathrin-coated vesicle formation include the EGFR pathway substrate clone 15 (Eps15), hepatocyte growth factor (HGF) regulated tyrosine kinase substrate (Hgs), syntapojanin, amphiphysin, and Epsin. The components of the endocytic machinery are large multi-domain proteins that possess multiple recruitment domains and are consequently involved in a wide spectrum of protein–protein and protein–phospholipid interactions. These endocytic proteins have functional homologues in yeast and *D. melanogaster*, suggesting that the basic mechanisms of endocytosis may be conserved across evolution. To address the possibility that phosphorylation of endocytic proteins may differentiate between constitutive and ligand-induced endocytosis, we will concentrate on two endocytic adaptors that are phosphorylated in response to EGF, namely Eps15 and Hgs (see Fig. 1).

5.1. Eps15

Eps15 was initially identified as major cytosolic substrate for ligand-activated EGFR [34]. An evolutionary conserved Eps15 homology (EH) domain is present in three copies in the amino terminus of Eps15. It was determined that a large number of EH domains present in a variety of endocytic proteins, both in yeast and in mammalian cells, recognize NPF (asparagine–proline–phenylalanine) tripeptides albeit with different sequence context preference [35]. It, therefore, comes as no surprise that Eps15 is endowed with a number of binding partners through the EH–NPF interaction. Some known interactions include Epsin, synaptojanin, and Numb, all of which are involved in endocytosis. Interestingly, the NPFXD sequence is a known internalization signal in yeast. It would be fascinating to explore the possibility that EH domain containing proteins, through their ability to bind NPF motifs, directly affect NPFXD containing receptors [36]. A class of peptides that contain two consecutive amino acids with aromatic side chains (FW or WW) are also recognized by some EH domains [37]. The central coiled-coil region of Eps15 is important for both homodimerization and heterodimerization of Eps15 with other endocytic proteins such as intersectin [38]. Downstream to the coil-coiled region, Eps15 contains multiple copies of the DPF tripeptide that binds to the amino-terminal 'appendage' region of AP2 [39]. The carboxyl terminal region of Eps15 also contains a proline-rich segment that is a target for SH3 domain-containing proteins. A recently identified UIM domain (see below, and Fig. 3) is also present in the carboxyl terminal region of Eps15. Eps15 is mainly cytosolic, although recently it has been shown to shuttle in and out of the nucleus [40]. A dramatic re-localization of Eps15 to the plasma membrane is detected upon EGF treatment [41]. It is of interest to point out that Eps15 is absent from clathrin-coated vesicles, but easily detectable in uncoated vesicles and endosomes, thus implying re-localization of Eps15 back into endocytic organelles. Expression of dominant negative mutant fragments of Eps15 [42], and microinjection of antibodies directed against Eps15 [43] demonstrate that Eps15 plays a critical role in both constitutive and regulated endocytosis. As previously mentioned, Eps15 was discovered as an EGFR pathway substrate and the major site of phosphorylation has been mapped to tyrosine 850. A Y850F mutant of Eps15 has dominant negative activity on the endocytosis of EGFR, but not of TfR [44]. Thus, the constitutive and ligand-induced pathways of endocytosis may utilize some of the same proteins, however, they utilize the proteins differently, contingent upon post-translational modifications such as tyrosine phosphorylation. Further analysis revealed that Eps15 undergoes mono-ubiquitylation in response to EGF [45]. The relevance of this observation will be addressed in greater detail when discussing the role of ubiquitin in the endocytic machinery.

5.2. Hgs

The identification of Hrs (renamed Hgs), similar to Eps15, resulted from its rapid and transient phosphorylation after cell treatment with several growth factors, including EGF [46]. Interestingly, tyrosine 334 has recently been identified as the site of EGF-mediated tyrosine phosphorylation [47]; the relevance of which awaits experimentation. Hgs is composed of several recognizable domains (Fig. 2): a VHS domain, a FYVE domain, a UIM domain, a proline-rich region, a coiled-coil domain, and a proline- and glutamine-rich carboxyl terminal region [48]. The FYVE domain confers binding to phosphatidylinositol 3-phosphate (PI3P) [49], and has been found to play a role in the localization of proteins such as EEA1 and Hgs to the early endosome. However, in the case of Hgs, the endosomal localization is also dependent upon the proline- and glutamine-rich C-terminal region [50]. The coiled-coil domain of Hgs mediates its interaction with sorting nexin 1 (SNX1). This interaction alters down regulation of EGFR, most likely by affecting trafficking downstream to internalization [51]. Other ligands of the coiled-coil domain include the signal transducing adapter molecule (STAM) and Hgs-binding protein (Hbp). The Hgs–STAM interaction leads to suppression of cytokine-mediated DNA synthesis [52]. Interestingly, EAST, the chicken homologue of STAM, forms a complex with EGFR in a ligand-dependant manner [53]. The Hgs–Hbp complex is important for receptor degradation [54]. An additional interactor of Hgs worth mentioning is the deubiquitylating enzyme UBPY [55]. Vps27p, the yeast homologue of Hgs, belongs to

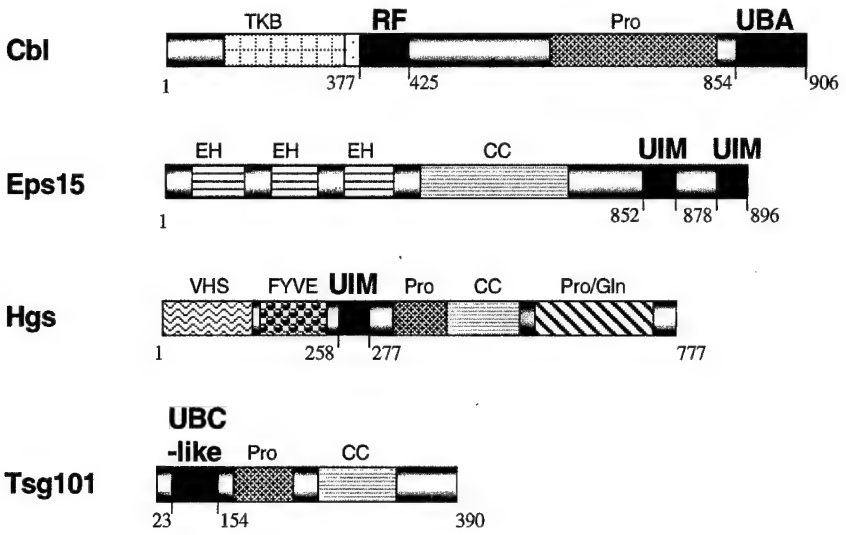

Fig. 2. Domain structures of proteins involved in ubiquitin-regulated endocytosis of surface receptors. The major recognizable domains of c-Cbl, Eps15, Hgs (Hrs), and Tsg101 are shown. Domains involved in protein ubiquitylation are indicated in bold along with the respective amino acid numbers. These include RING finger (RF), ubiquitin-associated domain (UBA), ubiquitin-interacting motif (UIM), and ubiquitin-conjugating enzyme E2 catalytic domain like (UBC-like). Other domains labeled are tyrosine kinase binding domain (TKB), proline-rich domain (Pro), proline- and glutamine-rich domain (Pro/Gln), Eps15 homology domain (EH), coiled-coiled domain (CC), Vps27/HRS/STAM domain (VHS), and phosphatidylinositol 3′-phosphate binding domain (FYVE).

class E Vps mutants which exhibit defective endocytic trafficking from the endosome to the vacuole [56]. Indeed, a similar function has been found for Hgs. Primary cultured, Hgs-deficient cells exhibit enlargement of TfR-positive early endosomes [57]. Furthermore, overexpression of Hgs leads to a conformational change in the early endosome [58] and accumulation of EGFR in vesicular aggregates [50,59]. Recently, the Hgs homologue in *D. melanogaster* was found to negatively regulate EGFR activity [60]. Intriguingly, by virtue of the recently discovered Hgs–clathrin interaction, Hgs has been implicated in the recruitment of clathrin to the endosomes [61]. An additional line of evidence linking Hgs to the endocytic machinery is the finding that Hgs not only interacts with Eps15, but negatively regulates Eps15 by affecting Eps15's association with α-adaptin [62]. Finally, we have mentioned that post-translational modifications such as tyrosine phosphorylation may differentiate the ligand-mediated endocytosis from the constitutive pathway. In support of this model, Urbe and colleagues demonstrated that internalization of EGFR into Hgs-containing endosomes is phosphorylation-dependent, and this event may release Hgs to a cytosolic pool [59]. By contrast with mammalian cells, yeast cells are unable to make use of tyrosine phosphorylation in ligand-induced endocytosis. However, it appears that ubiquitylation nevertheless plays this role in both mammals and yeast.

6. Lessons from yeast

Yeast homologues of clathrin and the AP2 complex exist, however, they are dispensable for endocytosis [63], and the function of these proteins in yeast endocytosis has remained elusive. Although internalization motifs have been identified in yeast polytropic G-protein-coupled mating factor receptors, recent findings indicate that ubiquitin functions alone to mediate internalization, and that some previously identified internalization sequences are primary sites for ubiquitin attachment. Internalization motifs include the sequence SINNDAKSS in the cytoplasmic tail of Ste2p [64], PEST [65], and NPFXD in the cytoplasmic tail of Ste3p [66]. After phosphorylation of the neighboring serine residues in the SINNDAKSS motif [67], the lysine residue is ubiquitylated, leading to receptor endocytosis [68]. Unraveling this mechanism led to the realization that monoubiquitylation of Ste2p is sufficient for internalization [69]. How does ubiquitin act as an internalization sequence? The precise answer to this question remains unresolved. However, the proper fold of the ubiquitin polypeptide seems required for the promotion of internalization [70], suggesting recognition by the endocytic machinery. An enigma that emerged from these studies relates to the ability of the internalization machinery to differentiate between ubiquitin that is receptor-bound and -free ubiquitin monomers.

Although appealing to generalize the difference between the constitutive pathway from the ligand-dependent route of endocytosis, an example from the mating pheromone receptors in yeast demonstrates the complexity of this issue. Two yeast pheromone receptors, Ste2p and Ste3p, are subject to both constitutive and ligand-induced endocytosis. Interestingly, in contrast to Ste2p, the more prominent mode of uptake for Ste3p is the constitutive pathway [65], which unlike Ste2p, is ubiquitylation-dependent and leads to degradation [71]. The ligand-dependent mode of endocytosis is

distinguished from the constitutive mode by the involvement of different cellular proteins that catalyze uptake. Instead of using the PEST internalization motif, ligand-induced uptake of Ste3p utilizes the NFPSTD internalization sequence. Furthermore, ligand-induced endocytosis leads to increased recycling of the receptor [72]. In contrast, ligand induction in the case of Ste2p results in ubiquitylation and an increase of vacuole-directed transport and degradation. In conclusion, the desire to mechanistically categorize endocytosis into a constitutive process that is dependent upon classical internalization signals and a regulated process that may depend on ubiquitylation may not be applicable to yeast receptors that participate in both pathways.

7. Involvement of protein ubiquitylation in ligand-stimulated receptor endocytosis

The traditional view of ubiquitylation focused on the role it plays in targeting misfolded proteins for degradation by the proteasome. However, it is now clear that ubiquitylation may target proteins to alternative destinations. The universality of ubiquitin conjugation demonstrates a fundamental resemblance to phosphorylation. Ubiquitin is covalently conjugated to proteins through the collective action of three enzymes [73]: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3) involved in target specificity. Like phosphorylation, ubiquitylation is a reversible process, with an activity analogous to that of phosphatases carried out by deubiquitylating enzymes (DUBs). Proteins may be phosphorylated or hyper-phosphorylated, and this distinction warrants functional differences. Similarly, ubiquitin may be conjugated to one lysine or to a number of lysines on a given molecule. On the other hand, the ubiquitylation machinery is distinct from the phosphorylation machinery in that ubiquitin may additionally be attached to the protein as a poly-ubiquitin chain. Phosphorylation can occur on either tyrosine, serine, or threonine residues leading to greater signaling diversity. Correspondingly, poly-ubiquitin chain formation can be linked by any of the internal ubiquitin lysines (e.g. lysine 48 or lysine 63). Among the many emerging functions of ubiquitylation, exocytosis and endocytosis appear to be regulated at more than one step by protein ubiquitylation. Here, we concentrate on the role played by c-Cbl, a ubiquitin ligase controlling ligand-stimulated endocytosis of RTKs (see Figs. 1 and 2).

8. Role of Cbl proteins in signal desensitization

The c-Cbl proto-oncogene was originally identified as a cellular form of v-cbl, a transforming protein of the Cas NS-1 retrovirus [74]. Sli-1, the *C. elegans* homologue of c-Cbl, is a negative regulator of signaling downstream to Let-23 in vulval development [75]. In concurrence, the *D. melanogaster* homologue, D-Cbl, suppresses the development of R7 photoreceptor cells [76], and functions as a negative regulator of an EGFR-dependent patterning pathway [77]. In mammals, the Cbl family consists of three members with highly conserved amino-terminal regions, c-Cbl, Cbl-b, and Cbl-3 [78].

c-Cbl is a large, ubiquitously expressed, predominantly cytoplasmic protein containing several distinct domains. The highly conserved N-terminal domain consists of the tyrosine kinase binding domain (TKB); named by virtue of its capacity to bind phosphotyrosine residues of multiple protein tyrosine kinases. The TKB itself is comprised of three domains, a four helix bundle, a calcium binding EF hand, and a Src homology 2 (SH2) motif. A short linker region recently found to be crucial for E3 activity and transformation [79] connects the TKB domain to the RING finger. The RING finger domain is the hallmark of Cbl's E3 ligase function (Fig. 3). The sequences that lie carboxy terminally to the RING finger contain an acidic domain of unknown function, and a proline-rich region, which contributes 15 potential SH3 domain binding motifs and also a serine-rich region with two 14-3-3 protein binding motifs. Finally, the most carboxy-terminal fragment contains a ubiquitin associated domain (UBA, see Fig. 3), and is also predicted to form a leuzine zipper capable of mediating homodimerization of Cbl.

c-Cbl has been described as a multivalent adaptor. Owing to its functional domains, c-Cbl binds to over 40 proteins involved in cellular regulation. The TKB domain binds phosphorylated cytoplasmic kinases such as ZAP-70 and several receptor tyrosine kinases such as EGFR. Ubiquitin-conjugating enzymes [31] and Sprouty [80], a protein found to negatively regulate EGFR signaling in *D. melanogaster*, bind to c-Cbl's RING finger domain. The proline-rich region is involved in interactions with numerous SH3-containing proteins such as Grb2, Src, and Cin85. Several phosphotyrosine residues of c-Cbl undergo phosphorylation by c-Src and other kinases and they form binding sites for c-Src itself [81], as well as for Vav, Abl, and the non-catalytic subunit of PI3K (see [82] for a recent review). The presence of multiple binding domains, and consequently a vast array of interacting partners, display multiple functionality. Indeed, c-Cbl has been found to play both a positive and a negative role in signaling. Bone re-absorption in osteoclasts is contingent upon positive signaling mediated by c-Cbl downstream to Src activation [83]. However, under a different cellular context c-Cbl can inhibit Src kinase activity [84].

The findings that c-Cbl is phosphorylated following EGF stimulation and forms an inducible complex with EGFR supported the genetic analyses of sli-1. Furthermore, an identical loss of function mutation from the *sli-1* gene in v-cbl abrogated EGFR association [85]. This mutation occurs within the SH2 domain of c-Cbl, which mediates phosphotyrosine-dependent interactions with substrates like EGFR. The leap forward in understanding the mechanism of c-Cbl action came from the observation that overexpression

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UBC
UBC1 (yeast)     DTK----VYH PNISSVTGAI CLDILK---- ---NAWSPV- ITLKSALISL -QALLQS-PE PNDPQDAEVA QHYLR-----
UBC7(human)      KTK----IYH PNID-EKGQV CLPVISA--- ---ENWKPA- TKTDQVIQSL -IALVND-PQ PEHPLRADLA EEYSK-----
Tsg101(human)    KPT---IKTG KHVD-ANGKI YLPYLH---- ----EWKHPQ SDLLGLIQVM IVVFGDE-PP VFSRPISASY PPYQA-----
Consensus        .........H PN.....G.. CL.IL..... .....W.P.. ......L... ...LL...P. ...P...... ..........
                                       *

UBA domain
Hr23A(human)     YETMLTEIMS M--GYERE-- RVVAALRASY NN-PHRAVEY LLT
Cbl(mouse)       LSSEIERLMS Q--GYSYQ-- DIQKALVIAH NN-IEMAKNI LRE
Consensus        .......L.. M..GF..... ....AL.... ......A... L..

RING finger
BRCA1 (human)    CPICLEL--- -----IKEPV STK-CDHI-- FCKFCMLKLL NQRKG----- -PSQCPLC
Cbl(human)       CKICAEN--- -----DKDVK IEP-CGHL-- MCTSCLTSWQ ESEG------ --QGCPFC
Consensus        C..C...... ..............C.H... .C..C..... .......... ....CP.C

UIM
S5A(human)       SADPELALAIRVSMEEQRQR
Hgs(human)       QEEEELQLALALSQSEAEEK
Eps15(mouse)     QEQEDLELAIALSKSEISEA
Consensus        ..D..L..A...S.......
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Fig. 3. Alignments of domains involved in linking endocytosis to ubiquitylation. Amino acid sequences of domains implicated in endocytosis and ubiquitylation (see Fig. 2) are shown, and consensus residues indicated in bold. The consensus sequences were derived from multiple alignments that are not presented. The asterisk marks the active cysteine residue of the UBC domain. Notice that Tsg101 contains a tyrosine (Y) residue at the corresponding site.

of c-Cbl leads to the ubiquitylation and degradation of EGFR [27] and the platelet-derived growth factor receptor (PDGFR) [86]. Soon thereafter it was demonstrated that c-Cbl is a negative regulator of additional RTKs such as the colony-stimulating factor 1 (CSF-1) receptor [87], ErbB-2 [88,89], and c-Met [90]. Finally, in vitro reconstituted ubiquitylation assays were employed to demonstrate that the c-Cbl's RING finger has intrinsic E3 ligase activity [29–31].

The mechanism of c-Cbl mediated downregulation of RTKs involves TKB binding to a specific phosphotryosine, such as tyrosine 1045 of EGFR [29], tyrosine 973 of CSF-1 receptor [91], tyrosine 1003 of the Met receptor [90], and tyrosine 1112 of ErbB-2 [88]. Alternatively, it was recently shown that a TKB binding mutant of EGFR (Y1045F) recruits c-Cbl indirectly through Grb2 [92]. After c-Cbl recruitment, EGFR phosphorylates tyrosine 371, which stimulates the ubiquitin ligase activity [29]. The site of c-Cbl function has been a matter of controversy for some time. Levkowitz et al. [27] demonstrated that c-Cbl expression will influence EGFR degradation but not internalization, a finding supported by another group [79]. In support of an endosomal role for c-Cbl, Burke et al. [20] have shown that association between EGFR and c-Cbl occurs in the early endosome. However, a Y1045F EGFR mutated in the c-Cbl docking site was found to be internalization defective [92]. Furthermore, expression of a dominant negative dynamin mutant which inhibits internalization did not abrogate EGFR ubiquitylation [93]. With the aid of alternative methods, de Melker et al. [94] have shown that indeed c-Cbl can associate with and ubiquitylate EGFR prior to clathrin-coated pit formation, however, they also concluded that c-Cbl and EGFR colocalize in the internal vesicles of the multi-vesicular body (MVB). Therefore, c-Cbl participates in the regulation of RTKs throughout the endocytic pathway. Cbl action on CSF-1 receptor is less controversial and occurs at the plasma membrane, as indicated by the delay of internalization in c-Cbl-knockout cells [87].

As previously mentioned, the addition of a single ubiquitin to a large number of plasma membrane proteins in yeast serves as an internalization signal [95]. The same may hold true for the Notch pathway in *D. melanogaster*. The transmembrane ligand Delta undergoes ubiquitin-mediated internalization [96]. Previously it was shown that Delta internalization results in trans-endocytosis of Notch and consequent activation of the Notch pathway [97]. It was additionally determined that expression of neuralized, the Delta ubiquitin ligase, increases Delta endocytosis and Notch signaling [98], thus illustrating that ubiquitylation at the plasma membrane governs internalization. In support of this model, the glycine receptor generated in *Xenopus* oocytes, is ubiquitylated prior to internalization [99].

Does ubiquitin function as an internalization motif in mammalian cells? CSF-1 receptor is not only ubiquitylated by c-Cbl prior to endocytosis [100,101], but internalization of CSF-1 is delayed in c-Cbl-knockout cells [87], signifying that ubiquitylation precedes internalization. A role for ubiquitylation in the regulation of the epithelial sodium channel at the plasma membrane was demonstrated [102]. Additionally, endocytosis of an internalization-defective interleukin-2 receptor (Tac) was restored when a single ubiquitin moiety was fused in-frame to the cytoplasmic region [103]. As previously mentioned, the question as to where c-Cbl mediates

EGFR ubiquitylation may be relevant to unraveling the components of ligand-induced EGFR internalization. It is plausible that in addition to phosphorylation of endosomal adaptors, a supplementary distinction that is not exclusive between constitutive and ligand-induced endocytosis is the ability of ubiquitin in the ligand-induced pathway to act as an inducible internalization signal. The ubiquitin sequence contains the motif $DQQRL^{43}I^{44}$ that may be similar to dileucine internalization motifs, such as the sequence DKQTLL. Indeed, in mammalian cells, mutations of either $leucine^{43}$ or the adjacent isoleucine resulted in the partial inhibition of endocytosis [103]. Studies in yeast revealed that this region is important as part of two hydrophobic patches critical for internalization. They concluded that two hydrophobic patches surrounding residues Phe^{4} and Ile^{44} mediate internalization [70]. Furthermore, a mutation of Phe^{4} that is unlikely to disrupt the structure of ubiquitin resulted in the inhibition of internalization, implying that this residue may be critical for protein–protein interactions [70]. In similarity to other transmembrane proteins, if EGFR is ubiquitylated at the plasma membrane and it is this ubiquitin that links the receptor to the endocytic machinery, an endocytic adaptor must recognize the internalization-destined modified receptor.

Interestingly, c-Cbl also contains a ubiquitin-associated domain (UBA) [104] that has been found to recognize specific ubiquitin conjugates [105–108]. As previously mentioned, the most carboxyl-terminal portion of c-Cbl is also predicted to form a leucine zipper capable of mediating homodimerization of c-Cbl, and the deletion of this region decreases both association of c-Cbl with EGFR and tyrosine phosphorylation of EGFR [109]. However, in vitro Cbl-b(N), which lacks the UBA domain, can still ubiquitylate EGFR. The understanding of this domain remains to be elucidated.

9. Do endosomal adaptors function as ubiquitin-recognizing receptors?

Recently, it was determined that a number of endocytic adaptors such as Eps15, Hgs, Epsin, and STAM share a structural domain called the UIM (ubiquitin interacting motif, see Fig. 3) [110]. The UIM was originally identified in a subunit of the regulatory complex of the proteasome that binds ubiquitylated lysozyme and free poly-ubiquitin chains [111]. Interestingly, it is the LAL(M)AL hydrophobic patch within the S5a UIM that is essential for multi-ubiquitin chain binding [112]. It has now been validated experimentally that indeed the UIM of Hgs binds ubiquitin [60]. It is of interest to point out that the Hgs' UIM was pulled down by monomeric ubiquitin and not by a multi-ubiquitin chain as required by the S5a subunit. It is therefore feasible that the UIM of Eps15, a protein recruited to the plasma membrane upon EGF treatment, recognizes the ubiquitin modification on the receptor and consequently acts as a 'connector' between the ubiquitylated cargo and clathrin-coated pit formation.

The viral oncogene product, v-Cbl, encompasses the amino terminal region of c-Cbl. It, therefore, binds EGFR through the TKB domain, but the lack of a RING finger leads to receptor binding, but no ubiquitylation. Whereas c-Cbl directs EGFR-loaded endosomes to degradation, v-Cbl directs the receptors to the alternative recycling pathway [27], a pathway associated with enhanced signaling and oncogenic transformation. Furthermore, it has been shown that ubiquitylation governs the transport of the interleukin-2 receptor from the early to the late endosomes [113]. In addition, recent work in yeast has demonstrated that ubiquitin is both necessary and sufficient for some membrane proteins to enter internal vesicles of multivesicular bodies [114]. Katzman and co-workers have demonstrated the mechanisms behind these findings. They found that Vps23p, a member of the ESCRT-1 complex, recognizes ubiquitylated cargo proteins at the endosome. It is the UBC-like domain (a domain with homology to ubiquitin-conjugating enzymes, see Figs. 2 and 3) in Vps23 that mediates recognition of ubiquitylated substrates [115]. Interestingly, the human homologue of Vps23p, Tsg101 also contains the UBC-like domain, and Tsg101 mutant cells display enhanced recycling of EGFR [116]. In conclusion, recognition of ubiquitin-conjugated cargo by specific endosomal proteins, such as Eps15, Hgs, Epsin and Tsg101, may be part of sorting machinery acting at the plasma membrane, as well as in the MVB.

10. Ubiquitylation in endocytosis is not restricted to cargo proteins

We have previously discussed how the ubiquitin system aids endocytosis at the level of both internalization and endosomal sorting. However, other observations have indicated that ubiquitylation is not restricted to cargo proteins. Research conducted on the growth hormone receptor (GHR) indicated that the ubiquitin machinery is required for internalization, although ubiquitylation of the receptor itself is not required [117]. As previously mentioned, Eps15 is mono-ubiquitylated upon EGF stimulation [45], and an Eps15 homologue in yeast, Pan1p, genetically interacts with Rsp5p, a HECT domain ubiquitin ligase [118]. Further confirmation that ubiquitylation plays an additional role in endocytosis is revealed by a finding in yeast. A mutant of Rsp5p, that lacks one of its domains retained the ability to ubiquitylate the Gap1 amino acid permease, but it was defective in permease downregulation [119]. These implications for a supplementary Rsp5p function was further verified when Dunn and Hicke [120] determined that Rsp5p ubiquitylates a yet unknown component of the endocytic machinery. Interestingly, although not part of the endocytic machinery per se, c-Cbl is also ubiquitylated. Recently, in *D. melanogaster*, the deubiquitylating enzyme fat facets was shown to de-ubiquitylate liquid facets, a homologue of the endocytic adaptor Epsin [121]. Further evidence is supported by the finding that β-arrestin, the adaptor protein of

the beta-adrenergic receptor is ubiquitylated, and this ubiquitylation is essential for receptor internalization [122]. Although several groups have determined that ubiquitylation of the endocytic machinery plays a positive role in endocytosis, some genetic evidence in *D. melanogaster* indicate that the opposite might be the case [123]. It is becoming clear that ubiquitylation plays an additional role in endocytosis, namely: regulation of the endocytic machinery. However, complete understanding of these findings remains an unsolved puzzle and may be illuminated upon in the future.

11. Concluding remarks

Unlike positive signaling, understanding molecular processes that underlie negative regulation of signal transduction is still in its infancy. Of relevance to the topic of this review, the contribution of endocytosis to receptor desensitization raises many unanswered questions. For example, while it is clear that c-Cbl is a primary regulator of tyrosine kinases, evidence derived from invertebrates implies functions unrelated to the RING finger and protein ubiquitylation. Likewise, the observed sustained partnership between EGFR and endocytic proteins like c-Cbl and Hgs throughout the endocytic pathway suggests that sorting events recur while a receptor is translocated through successive vesicular compartments. The type of ubiquitin modifications, namely mono- or poly-ubiquitylation, as well as the type of ubiquitin branching and the role played by ubiquitin-like molecules remain obscure. Similarly, deubiquitylation reactions seem to play an important role in signaling, and this notion is supported by genetic evidence. However, only little information is available on the role of deubiquitylation in mammalian signaling. Lastly, as ubiquitylation plays a pivotal role in desensitization of oncogenic kinases, it is plausible that future therapeutic strategies will harness the ubiquitylation system. For instance, cancer immunotherapy using monoclonal antibodies to ErbB-2, the closest relative of EGFR, seems to enhance endocytosis of the oncogenic receptor, but the role of ubiquitylation in antibody-induced endocytosis of membrane receptors is unclear at present. In a similar way, under stress conditions surface receptors are cleared through the action of the Hsp90/Hsp70 chaperones, and while ubiquitylation is involved in stress-induced endocytosis, mechanisms governing this pathway of receptor down-regulation are yet unclear. Like immunotherapy, stress conditions may have clinical ramifications in chemo- and radio-therapy of cancer. Clearly, detailed understanding of the role played by protein ubiquitylation in the different pathways of endocytosis is lacking, but the emerging data will likely lead to improved ways to combat cancer.

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Molecular mechanisms underlying ErbB2/HER2 action in breast cancer

Daniel Harari[1] and Yosef Yarden*,[1]

[1]*Department of Biological Regulation, the Weizmann Institute of Science, Rehovot 76100, Israel*

Overexpression of ErbB2, a receptor-like tyrosine kinase, is shared by several types of human carcinomas. In breast tumors the extent of overexpression has a prognostic value, thus identifying the oncoprotein as a target for therapeutic strategies. Already, antibodies to ErbB2 are used in combination with chemotherapy in the treatment of metastasizing breast cancer. The mechanisms underlying the oncogenic action of ErbB2 involve a complex network in which ErbB2 acts as a ligand-less signaling subunit of three other receptors that directly bind a large repertoire of stroma-derived growth factors. The major partners of ErbB2 in carcinomas are ErbB1 (also called EGFR) and ErbB3, a kinase-defective receptor whose potent mitogenic action is activated in the context of heterodimeric complexes. Why ErbB2-containing heterodimers are relatively oncopotent is a function of a number of processes. Apparently, these heterodimers evade normal inactivation processes, by decreasing the rate of ligand dissociation, internalizing relatively slowly and avoiding the degradative pathway by returning to the cell surface. On the other hand, the heterodimers strongly recruit survival and mitogenic pathways such as the mitogen-activated protein kinases and the phosphatidylinositol 3-kinase. Hyper-activated signaling through the ErbB-signaling network results in dysregulation of the cell cycle homeostatic machinery, with upregulation of active cyclin-D/CDK complexes. Recent data indicate that cell cycle regulators are also linked to chemoresistance in ErbB2-dependent breast carcinoma. Together with D-type cyclins, it seems that the CDK inhibitor $p21^{Waf1}$ plays an important role in evasion from apoptosis. These recent findings herald a preliminary understanding of the output layer which connects elevated ErbB-signaling to oncogenesis and chemoresistance. *Oncogene* (2000) **19,** 6102–6114.

Keywords: carcinoma; growth factor; signal transduction; tyrosine kinase; cyclin D1; Waf1

Introduction

Neu/Her2, also known as ErbB2, was first discovered as a potent oncogenic mutant, when isolated from independent neuroglioblastomas or Schwannomas that developed in carcinogen-treated rats (Schechter *et al.*, 1984). Although this and other ErbB2 mutations are rarely, if at all found in human cancers, wild-type ErbB2 has been often found to be either amplified at the genomic level and/or found to be overexpressed at the protein level. In the majority of cases overexpression correlates with tumor chemo-resistance and poor patient prognosis (reviewed in Hynes and Stern, 1994; Klapper *et al.*, 2000a). This subject has been most extensively addressed in the domain of breast cancer biology, in which ErbB2 is amplified at a 20–30% incidence, and where therapies to subvert ErbB2 expression are now impacting in the clinic.

The first part of this review will address how ErbB2, a member of a complex signaling network, normally functions; an understanding of which is necessary before it can be attempted to elucidate the molecular mechanisms it exploits in cancer. How Her2/ErbB2 overexpression translates into signals that potentiate dysregulated growth, oncogenesis, metastasis and possibly also chemoresistance, particularly in relation to breast cancer, will then be addressed. Not surprisingly, ErbB2 overexpression hyperactivates components of the cell cycle machinery; changes which are also linked to resistance against apoptosis-inducing therapeutic agents. These recent advances towards a clairvoyant understanding of ErbB2 function in cancer, as well as advances in our understanding of the mechanism in which ErbB2-blocking antibodies (Trastuzumab; Herceptin) can in some but not all cases, help facilitate regression of ErbB2-overexpressing tumors will be discussed. This review offers a biochemical and semi-clinical perspective. Reviews directly based on clinical aspects are found elsewhere (e.g. *Seminars in Oncology*, Volume 26, No. 4. Suppl 12 (1999) Whole Issue; Burris, 2000; DiGiovanna, 1999; Klapper *et al.*, 2000a)). Additionally, a commercially-backed internet site devoted to the use of Herceptin is currently accessible (http://www.prous.com/herceptin).

A mechanistic understanding of ErbB2 function

The ErbB receptor family

ErbB2 is a member of the membrane-spanning type I receptor tyrosine kinase family, comprising four closely related family members, in which ErbB1 (also known as the EGF receptor) was the first to be molecularly cloned (Ullrich *et al.*, 1984). In common with many other growth factor receptors, members of the ErbB family dimerize upon ligand stimulation and transduce their signals by subsequent autophosphorylation catalyzed by the receptor cytoplasmic tyrosine kinase activity, which results in recruitment of an array of downstream signaling cascades (reviewed by Hunter, 2000). The type and amplitude of activated downstream signaling cascades are a co-function of which receptors are expressed by a particular cell, the number of receptors expressed, and the amount and type of ligand that stimulates the cell (Burden and Yarden, 1997; Holland *et al.*, 1998; Schlessinger and Ullrich, 1992).

*Correspondence: Y Yarden

The heterodimerization model

Although active homodimers can naturally form both for ErbB1 and for ErbB4, ligand-stimulated heterodimer formation is often the norm. In particular, ligand-stimulated heterodimerization is a prerequisite for active signaling for both the ErbB2 and the ErbB3 receptors; ErbB2 binds no known ligand with high affinity, and upon stimulation can only be recruited as a co-receptor with another ErbB member (Klapper *et al.*, 1999). In other words, no known ligand can activate ErbB2 homodimers. Conversely, ErbB3 binds a number of ligands with high affinity, but harbors a defective tyrosine kinase, and thus requires co-recruitment with another ErbB-member to be transactivated (Carraway and Cantley, 1994). A consequence of the 'heterodimerization model' is that a particular receptor essentially requires two other signaling components to initiate its activation; namely a co-receptor and a high affinity ligand. Evidence to support this model are described below. The concept of this model is critically important for an understanding of the mechanisms underlying at least early stages of ErbB2-related cancers as it dictates that ErbB2 is not activated in isolation, but rather in concert with at least two other components of its signaling network.

Genetic evidence to support the heterodimerization model Developmental genetic studies provide evidence to indirectly support the heterodimerization model. Three examples are described.

(1) Mouse embryos devoid of functional ErbB2 die at mid-gestation due to a deficiency of ventricular myocardial trabeculation (Lee *et al.*, 1995). A common defect was similarly found in two other mutant mouse strains; one with a targeted disruption of ErbB4 and the other with a disruption of the ErbB-ligand Neuregulin-1 (NRG1) (Gassmann *et al.*, 1995; Meyer and Birchmeier, 1995). Cells expressing NRG1 are juxtaposed to the developing cells of the ventricular myocardium which co-express ErbB2 and ErbB4, demonstrating paracrine cross-talk between these adjacent tissues. Thus there is an essential requirement for these three genes in the same developmental process, consistent with the notion that NRG1-driven ErbB2-ErbB4 heterodimers are essential for heart trabeculae formation.
(2) NRG1-, ErbB2- and ErbB3-mutant mice all demonstrate a common deficit in precursor Schwann cell development, thus implicating a requirement of NRG1-driven ErbB2-ErbB3 dimers at this developmental checkpoint (Gassmann *et al.*, 1995; Lee *et al.*, 1995; Meyer and Birchmeier, 1995).
(3) Genetic studies implicate essential roles for ErbB1 in both homo- and heterodimeric complexes. Mice harboring a kinase defective mutant of ErbB1, thus unable to form active ErbB1 homodimers but able to form active heterodimers, exhibit a rather mild phenotype which is very similar to that for mice mutant for the ErbB ligand TGF-alpha (Fowler *et al.*, 1995; Luetteke *et al.*, 1994; Mann *et al.*, 1993; Walker *et al.*, 1998). However ErbB1 knockout mice exhibit extremely severe embryonic abnormalities, implicating an additional requirement for ErbB1 heterodimeric complexes in some developmental processes (Sibilia and Wagner, 1995; Threadgill *et al.*, 1995).

Biochemical evidence for the heterodimerization model Cell-lines devoid of endogenous ErbB receptor expression have provided a 'clean' experimental system in which to study ErbB function. In these and other cells, stable expression of different ErbB receptors, either singly, or in combinations, has allowed the analysis of ligand-induced ErbB signaling through different homo- and heterodimeric combinations (Alimandi *et al.*, 1997; Cohen *et al.*, 1996a; Pinkas-Kramarski *et al.*, 1996a; Riese *et al.*, 1995; Zhang *et al.*, 1996). Likewise, depletion of ErbB2 expression at the cell surface using either intracellular antibodies (Beerli *et al.*, 1994) or specific ribozymes (Czubayko *et al.*, 1997; Juhl *et al.*, 1997) has allowed the examination of the role of the co-receptor in signaling and tumorigenesis. Importantly, ErbB2 or ErbB3 singly expressed in cells devoid of other ErbBs cannot be activated by ErbB-ligands, even at very high ligand concentrations. However, in the presence of a co-receptor, ErbB2 and ErbB3 promote strong intracellular signaling (Harari *et al.*, 1999; Pinkas-Kramarski *et al.*, 1996a, 1997). Heterodimer formation can be detected by co-immunoprecipitation, trans-phosphorylation and ligand cross-linking analyses. Ligand-induced physical interactions between ErbB proteins was first demonstrated for ErbB1-ErbB2 complexes and later extended for other receptor combinations. In fact, such heterodimeric complexes have been found for every ErbB receptor combination (Tzahar *et al.*, 1996). Thus from a family of four receptors, 10 complexes can be formed: four homodimers and six heterodimers. It is worth mentioning that this complexity evolved relatively late in evolution, as worms and insects express only one ErbB-like receptor (Perrimon and Perkins, 1997). Thus, replacement of the invertebrate linear pathway of ErbB with a mammalian signaling network may represent the need to increase the diversity of this network's output.

Ligands that co-activate ErbB2

Ten distinct mammalian genes, some with numerous alternative splice variants, encode either the 'EGF receptor ligands' that bind ErbB1, or the 'Neuregulins' that can bind ErbB3 and/or ErbB4, with ErbB2 as a co-receptor. This classification system although valid, is somewhat over-simplistic. To different extents, most ligands have the capacity to bind with high affinity to more than one receptor (Figure 1B). In addition, all ErbB-ligands readily activate ErbB2 in combination with the appropriate high affinity co-receptor. This activation is often of higher affinity and biological potency than complexes lacking ErbB2 (Graus-Porta *et al.*, 1995, 1997; Harari *et al.*, 1999; Karunagaran *et al.*, 1996; Klapper *et al.*, 1999; Riese *et al.*,1996; Tzahar *et al.*, 1996; Wang *et al.*, 1998). Mammalian ErbB-ligands are mostly membrane spanning, possessing a single transmembrane domain with resultant cytoplasmic and extracellular components. They are structurally divergent, with only a single common trait at the primary structural level; they all encode an extracellular EGF-

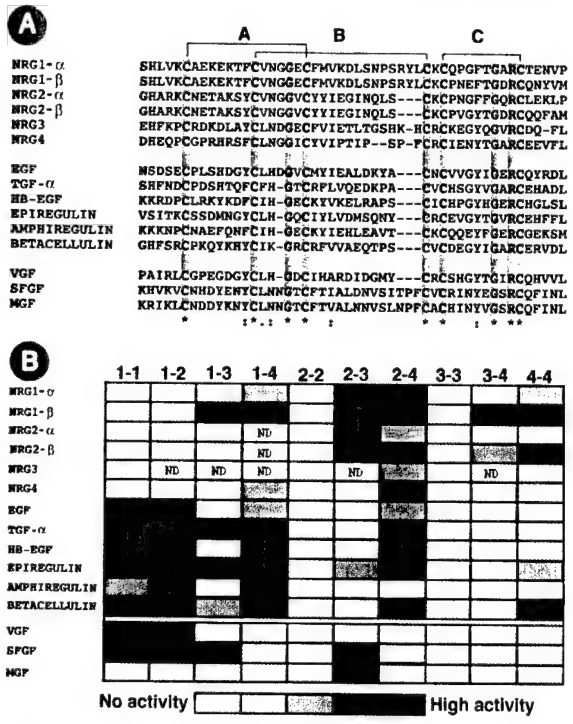

Figure 1 Multiplicity of ErbB ligands. (**a**) Alignment of the EGF-like domains of mammalian and viral ligands. ErbB-receptor binding is conferred by the EGF-like domains found for all members of this ligand family, their primary sequences given here. All sequences of the mammalian ligands are human in origin, with the exception of NRG-3 and NRG-4 (mouse). The three pox virus-encoded ligands (bottom) are shown separate from the mammalian ligands. Sequences were aligned using ClustalX software (default parameters). Three predicted disulfide bridges (Cys 1-3, Cys 2-4, Cys 5-6) are labeled as loops A, B and C. Primary references for these sequences are described elsewhere (Harari *et al*., 1999; Tzahar *et al*., 1998). Symbols at the bottom represent canonical residues (*), highly conserved amino acids (:), or conserved residues (.). (**b**) Ligand activation profiles for different ErbB-receptor combinations. Ten possible dimeric complexes of ErbB receptor proteins are represented (columns). The degree of activation by a particular ligand to a receptor combination is indicated by a shaded scale. Activation was based upon physiological mitogenic readouts wherever possible, with exception of NRG-3 where presumed activation was extrapolated from binding data. This composite was derived from numerous sources (Alimandi *et al*., 1997; Crovello *et al*., 1998; Graus-Porta *et al*., 1997; Harari *et al*., 1999; Jones *et al*., 1999; Lenferink *et al*., 1998; Pinkas *et al*., 1998; Pinkas-Kramarski *et al*., 1998; Riese *et al*., 1995, 1996, 1998; Wang *et al*., 1998; Zhang *et al*., 1997). (ND) To our knowledge not determined

like domain, which confers ligand-binding capacity (Jacobsen *et al*., 1996). The characteristic six cysteine residues and conserved spacing of the EGF-like domain predicts the existence of three disulfide bridges, denoted as A, B and C (Figure 1A). The genes NRG1 and NRG2 are unusual in that they harbor two alternate tails of the EGF-like domains (alpha and beta isoforms) which have arisen as a result of exon duplication and differential exon usage (Carraway *et al*., 1997; Chang *et al*., 1997; Wen *et al*., 1994). Currently the role of the transmembrane topology of the precursors of ErbB ligands is not well understood. Whereas experiments performed with pro-TGF-alpha implied that the precursor can interact with a receptor expressed at the surface of a neighboring cell (juxtracrine signaling, reviewed in Massague and Pandiella, 1993), more recent analyses performed in insect and in mammalian cells suggest that transmembrane ligands may be inactive. Proteolytic cleavage of the extracellular domain, resulting in release of the active EGF-like domain seems to be required for ligand activation (Loeb *et al*., 1998; Schweitzer *et al*., 1995).

ErbB2 overexpression potentiates signaling by evading inactivation processes

Ectopic overexpression of ErbB2 to the level observed in breast and other types of tumors enhances tumorigenicity in model systems (Di Fiore *et al*., 1987; Hudziak *et al*., 1987). The mechanism underlying the tumorigenic action of an overexpressed ErbB2 may relate to the relatively high basal autophosphorylation activity of this receptor-like tyrosine kinase (Lonardo *et al*., 1990). Thus, by overexpressing ErbB2 at the cell surface, homodimers may spontaneously form, in analogy to the carcinogen-activated form of the rodent Neu/ErbB2 whose mutation promotes dimerization (Weiner *et al*., 1989). Alternatively, overexpression of ErbB2 may increase its availability for heterodimer formation when a ligand binds to its direct receptor. Consistent with a heterodimerization model, the transforming action of the rodent mutant depends on a co-expressed ErbB protein (Cohen *et al*., 1996b). In addition, the transforming ability of ErbB2 is significantly enhanced when co-expressed with either ErbB1 or ErbB3, but in both cases a respective ligand seems essential (Kokai *et al*., 1989; Wallasch *et al*., 1995). Thus, an overexpressed ErbB2 may promote carcinogenesis primarily in the context of a ligand-driven heterodimer.

Analyses of the repertoire of signaling molecules that associate with ErbB2 upon autophosphorylation revealed no unique substrate which would explain the transforming action. However, the transforming mutant of ErbB2, which serves as a model system for human cancer, strongly interacts with both the MAPK (Ben-Levy *et al*., 1994) and the PI3K pathways (Peles *et al*., 1992). These observations support that cell proliferation and cell survival are activated by ErbB2 through these respective biochemical pathways. Despite the absence of an ErbB2-specific substrate, its signaling ability appears unique in that it is less accessible to normal inactivation processes. For example, signaling through MAPK pathways is significantly prolonged and enhanced in cells overexpressing ErbB2, as opposed to cells whose level of expression is relatively low (Karunagaran *et al*., 1996). Normally, signaling by growth factor receptors is rapidly inactivated by several mechanisms that safeguard short signals. Dissociation of ligand-receptor complexes, dephosphorylation of the activated receptor, rapid internalization through clathrin-coated pits and degradation of active receptors in lysosomes, ensure rapid termination of signals. With the exception of receptor dephosphorylation, these processes are slowed down when ErbB2 is overexpressed.

(i) *ErbB2 decelerates ligand dissociation rates*: Although ErbB2 binds no known ligand with high affinity, when overexpressed it elevates the affinity of EGF (Wada *et al*., 1990) and

neuregulins (Peles *et al.*, 1993; Sliwkowski *et al.*, 1994; Tzahar *et al.*, 1996) to their receptors. The mechanism of increased affinity to multiple ligands can be related to a specific site upon ErbB2, as a class of monoclonal antibodies directed to the extracellular domain of the oncoprotein could accelerate dissociation of several ligands (Klapper *et al.*, 1997). Indeed, overexpression of ErbB2 affects the rate of ligand release from active dimers, but the rate of ligand association with the direct receptor is not affected (Karunagaran *et al.*, 1996). That dimeric receptors bind ligands with higher affinity than monomeric receptors is implied by experiments that made use of mutation-driven dimers of ErbB proteins (Ben-Levy *et al.*, 1992). In conclusion, the stability of receptor dimers, and especially heterodimers with ErbB2, is higher than that of other receptor combinations.

This can explain why overexpression of ErbB2 can prolong intracellular signaling, but it leaves open the exact mechanism of ErbB2 involvement. A combination of biochemical, biophysical and mutation mapping analyses have found that ErbB2 can act as a low-affinity and broad-specificity receptor for ErbB-ligands, in contrast to high-affinity binding conferred by other receptors, which has resulted in the inception of a bivalency model of ligand-receptor interaction (Tzahar *et al.*, 1997). In this model, each ErbB-ligand harbors both a high and a low-affinity receptor-binding site. In the case of NRG1, the high-affinity site maps to the N-terminal region of the EGF-like domain, whereas a low-affinity receptor binding site resides on the C-terminal region of the EGF-like domain (Barbacci *et al.*, 1995; Jones *et al.*, 1999; Pinkas-Kramarski *et al.*, 1996a; Tzahar *et al.*, 1996). That ErbB2-containing receptor complexes tend to be the most potent (Graus-Porta *et al.*, 1997; Harari *et al.*, 1999; Jones *et al.*, 1999; Klapper *et al.*, 1999; Riese *et al.*, 1996; Wang *et al.*, 1998), suggests that the bivalency model applies to other ligand-receptor complexes. Therefore, according to this model, one aspect of ErbB2's potent oncogenicity is its preferential binding as a low-affinity receptor to different ErbB-ligands.

(ii) *ErbB2 is endowed with a relatively slow rate of endocytosis*: Chimeric and wild-type ErbB1 or ErbB2 receptors were employed to demonstrate that the carboxyl-terminus of ErbB2 can reduce 3–4-fold the rate of internalization of ErbB1 (Sorkin *et al.*, 1993). This results in impaired downregulation and degradation of ErbB2, and may explain why heterodimers with ErbB2 initiate relatively long-lived intracellular signals. Likewise, comparative analyses of the rates of internalization of EGF and NRG1 revealed dramatic differences: EGF underwent much more rapid internalization than NRG1, especially when homodimers of ErbB1 were involved (Baulida *et al.*, 1996; Pinkas-Kramarski *et al.*, 1996b). The mechanism responsible for inefficient entrapment of ErbB2 by the clathrin-coated pit is still unknown but it may relate to reduced ability to bind the clathrin-associated protein AP-2.

(iii) *ErbB2-containing heterodimers are destined for recycling and their degradation is relatively slow*: When ErbB2 is co-expressed with ErbB1, its presence cannot affect the rate of EGF internalization. However, ErbB2 overexpression enhances the rate of recycling of ErbB1 to the cell surface, and at the same time, reduces lysosomal targeting of the EGF-receptor (Lenferink *et al.*, 1998; Worthylake *et al.*, 1999). Because recycling back to the cell surface is primarily a default mechanism, it seems that ErbB2 affects only the rate of delivery to the lysosome. This process is controlled by prior ubiquitination of internalized receptors, a modification that takes place in the early endosome. The mechanism of ubiquitination of ErbB1 has been recently attributed to the activity of c-Cbl, an ortholog of Sli1, the major negative regulator of ErbB signaling in worms (Jongeward *et al.*, 1995). When ErbB1 is activated by EGF, tyrosine 1045 of the carboxy terminus is phosphorylated and recruits c-Cbl. The latter is a ubiquitin ligase that uses a RING finger domain to transfer ubiquitin molecules from an enzyme intermediate to the substrate, ErbB1 (Levkowitz *et al.*, 1998, 1999). By contrast with ErbB1, coupling of ErbB2 to c-Cbl is relatively weak. Although tyrosine 1112 of ErbB2 seems to act as a docking site, ubiquitination and lysosomal targeting of the oncoprotein by c-Cbl are relatively inefficient (Klapper *et al.*, 2000b). In conclusion, lysosomal targeting of ErbB2 is impaired and in the context of a heterodimer ErbB2 can drag ErbB1 to the recycling pathway.

In summary, several distinct mechanisms allow prolonged retention of ErbB2 at the cell surface (Figure 2), thereby extending the duration of signaling by its heterodimeric partners. Especially important is ErbB3, as this kinase-defective receptor is expressed at moderately high levels in most types of human epithelium, and its ectopic co-expression with ErbB2 confers a transformed phenotype (Alimandi *et al.*, 1995; Wallasch *et al.*, 1995). Unlike other receptors, ErbB3 is constitutively recycled to the cell surface and its rate of internalization and degradation are very slow (Baulida *et al.*, 1996; Waterman *et al.*, 1998). The mechanisms underlying mitogenic superiority of the kinase-defective member of the ErbB family were attributed to enhanced interaction with the PI3K pathway and avoidance of the c-Cbl-mediated degradative fate (Waterman *et al.*, 1999).

ErbB2 overexpression in breast cancer

ErbB2

The oncoprotein is overexpresed in 20–30% of breast tumors but particularly so (~90%) in comedo forms of Ductal Carcinoma *in situ* (DCIS), a malignant ductal carcinoma that has not progressed beyond the basement membrane barrier (Barnes *et al.*, 1992; Slamon *et al.*, 1987; van de Vijver *et al.*, 1988). However, even after progression to invasive disease, a correlation with the DCIS subtype and ErbB2 overexpression is maintained (Barsky *et al.*, 1997; Brower *et*

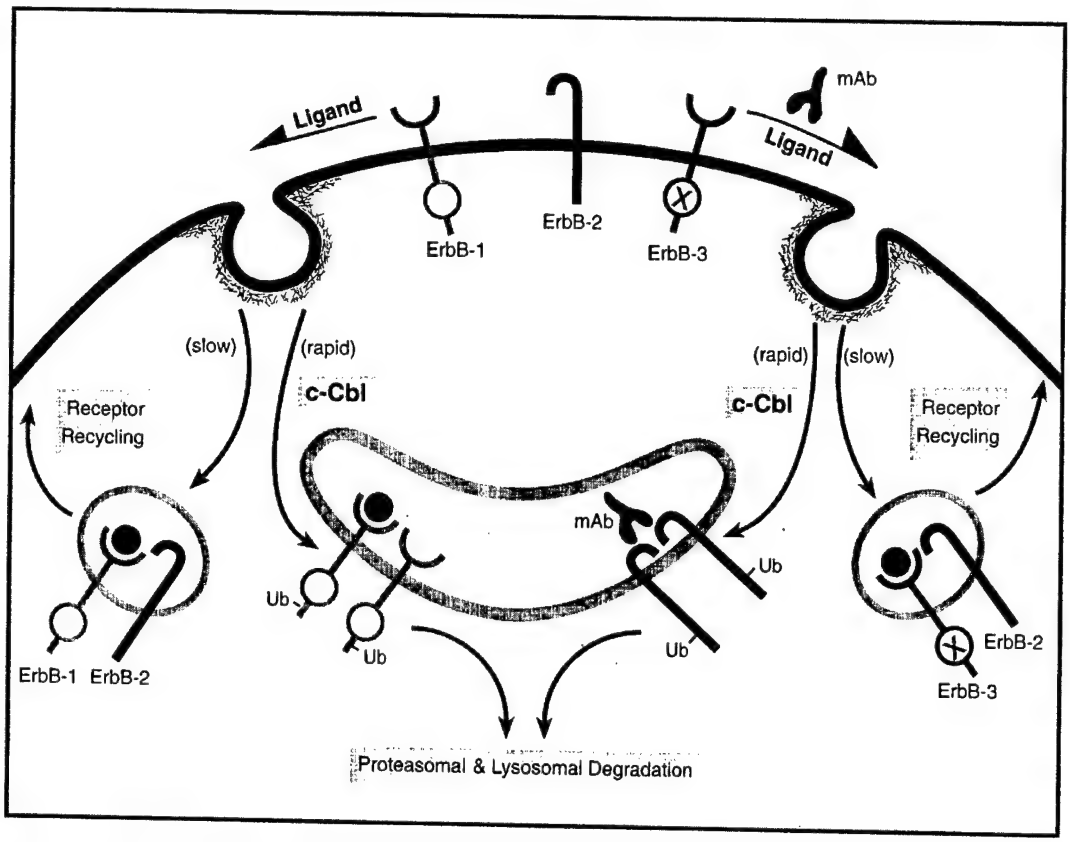

Figure 2 Alternative intracellular sorting of ErbB proteins. Binding of a ligand or a monoclonal antibody to ErbB proteins results in accelerated removal from the cell surface through coated regions of the plasma membrane. Invagination of clathrin-coated regions is followed by receptor delivery to endosomes. Subsequently, endocytosed receptors may be targeted to lysosomal degradation. Sorting to the lysosome is mediated by tyrosine phosphorylation and by c-Cbl, a ubiquitin (Ub) ligase that tags incoming receptors with polyubiquitin tracts. As an alternative route, endocytosed receptors may be recycled back to the cell surface, but in either case, the ligands are degraded. Which endocytic route is taken is determined by the identity of the receptor, its dimeric partner and to some extent by the identity of the ligand. Note that homodimers of ErbB1 are destined to lysosomal degradation, whereas ErbB1-ErbB2 heterodimers tend to recycle. Recycling is even more prominent in the case of ErbB3, a receptor whose kinase is defective (marked by a cross) and whose coupling to c-Cbl is inefficient

al., 1995). Importantly, high ErbB2 predicts lower disease-free and overall survival in both lymph node negative and particularly in lymph node positive tumors, indicating a functional role of ErbB2 in breast cancer (see Klapper *et al.*, 2000a and references therein). As ligand-dependent stimulation of ErbB2 essentially requires the recruitment of a co-receptor for its activation, another ErbB receptor is thus implicated in ErbB2-dependent cancers. Univariate and co-expression analysis of different ErbBs give no definitive clues as to a single receptor partner that co-activates along with ErbB2.

ErbB1

This receptor seems to be a prime ErbB2 co-receptor candidate; its overexpression or amplification correlates inversely with Estrogen Receptor status, is often expressed in invasive ductal carcinomas and correlates with poor prognosis especially in node negative and perhaps also in node positive patients (Fox *et al.*, 1994; Harris *et al.*, 1992; Nicholson *et al.*, 1991; Torregrosa *et al.*, 1997; VanAgthoven *et al.*, 1995). These phenotypes often overlap with that found for univariate ErbB2 overexpression studies. However, co-expression analyses did not find a significant positive or negative correlation between ErbB1 and ErbB2 overexpression. Cases in which co-overexpression did occur were associated with worse disease free survival prognosis than by either risk factor alone (Torregrosa *et al.*, 1997). These results suggest that ErbB1 plays a similar but not necessarily inter-dependent role in ErbB2-related breast cancers, although their synergy in a subset of tumors does indirectly implicate co-receptor interaction and a more aggressive phenotype.

ErbB3 and ErbB4

Different studies give conflicting reports as to the clinical significance of ErbB3 or ErbB4 expression in breast cancer. If at all, there is a tendency for an observed increase in tumor size, a positive correlation with Estrogen Receptor status and at least for ErbB3, an increased survival prognosis (Bacus *et al.*, 1996a; Gasparini *et al.*, 1994; Knowlden *et al.*, 1998; Lemoine *et al.*, 1992; Quinn *et al.*, 1994; Srinivasan *et al.*, 2000; Travis *et al.*, 1996; Vogt *et al.*, 1998). However, most tumors express either ErbB3 or ErbB4 making statistical assessment of certain tumor subtypes difficult on the basis of differences in receptor expression status

alone. It remains an open question if ErbB3 and/or ErbB4 play a significant role in the co-activation of ErbB2 in breast cancers or not. For example, in a pilot-sized study, two-thirds of patients with ErbB2 positive DCIS tumors also exhibited ErbB3 expression (Naidu *et al.*, 1998). As ErbB3 is often expressed in other tumor types, it is difficult to assess if there is a consequential meaning to the co-expression of ErbB2 and ErbB3 or not. It is relevant that initial studies performed with other types of tumors attribute clinical significance to co-expression of ErbB proteins. For example in oral squamous cell cancer co-expression of ErbB2 with ErbB1 or ErbB3 was significant and it critically improved the predicting power (Xia *et al.*, 1999). Future studies on stratified breast tumors may reveal similar relationships.

ErbB ligands

Which of the many ErbB ligands play a critical role in breast cancer is difficult to define as the stroma of the mammary gland is normally enriched for a number of ligands, albeit with different spatial-temporal profiles (Luetteke *et al.*, 1999; Normanno *et al.*, 1994; Herrington, 1997 #4534). Transgenic mouse models overexpressing for example either TGF-alpha or NRG1 under the control of the MMTV promoter demonstrate an accelerated incidence of mammary carcinoma (Krane and Leder, 1996; Sandgren *et al.*, 1990). Tumors usually arise only after a number of months in these mice and are focal in nature, indicating that independent additional genetic events are required for tumorigenesis to occur.

A move to growth factor independence?

Growth factor dependence dictates a requirement for localized ligand stimulation as well as an ErbB co-receptor to maintain ErbB2 signaling for a particular cell. Mammary stroma are enriched for numerous ErbB ligands; some are upregulated in tumors (Ciardiello *et al.*, 1991; de Jong *et al.*, 1998; Lundy *et al.*, 1991; Panico *et al.*, 1996). However, metastatic invasion of tumors to locations such as the lymph nodes, which are presumed poor in ErbB ligand expression may indicate a switch from factor dependence to independence. Two separate mechanisms which can support a factor independent model in ErbB2 or ErbB1 overexpressing tumors are described here. In one model, a naturally but rarely transcribed splice variant of ErbB2 with an in-frame deletion of 16 amino acids in the extracellular domain has been cloned, which is oncogenic in nature and can induce ligand-independent activation of ErbB2 (Siegel *et al.*, 1999). This novel transcript was estimated to be transcribed rarely; from 2–5% of ErbB2 mRNA in total. However, assuming that heterodimers of this gene product with the wild type counterpart result in ligand-independent activation, then potent activation can take place in tumors where ErbB2 is amplified. A second model derived from numerous studies of EGF Receptor (ErbB1) signaling, demonstrate that the EGFR can be trans-activated by a number of heterologous signal transduction pathways. Signaling through different G-protein-coupled-receptors (GPCRs), the growth hormone receptor, interleukin receptors, or voltage-gated calcium channels can all result in the trans-activation of ErbB1 and its downstream components (reviewed by Carpenter, 1999; Zwick *et al.*, 1999). The mechanism of GPCR-mediated trans-activation of ErbB1 was partially solved, where it was found that activation of GPCRs resulted in the cleavage of the ligand HB-EGF from its membrane bound inactive precursor, through the action of a metalloproteinase (Prenzel *et al.*, 1999). This model therefore does not exactly demonstrate ErbB-ligand-independence, but rather the activation of an autocrine loop.

The yin and yang of estrogen receptor and ErbB expression

A negative correlation between ErbB2 and estrogen receptor expression

Numerous studies have shown an intriguing interrelationship between ErbB2 and estrogen receptor (ER) expression and to a lesser extent, progesterone receptor (PR) expression in breast cancer. With exception, there is a strong, highly significant inverse relationship between estrogen receptor expression status and either ErbB2 or ErbB1 overexpression (Battaglia *et al.*, 1988; Roux *et al.*, 1989). Expression of these markers are of clinical significance. ER-negative tumors are more likely to express ErbB1 and/or ErbB2, to be more aggressive and infiltrating in nature and are less often expressed in DCIS. All these trends reversed in patients with ER-positive tumors. A common protocol for patients presented with ER-positive tumors, is to treat them with 'anti-estrogens' such as Tamoxifen. Not surprisingly, patients with ErbB2 overexpressing tumors respond poorly to Tamoxifen therapy (Borg *et al.*, 1994; Carlomagno *et al.*, 1996; De Placido *et al.*, 1998; Houston *et al.*, 1999; Newby *et al.*, 1997). However, it seems that ErbB2 signaling can override the tumor-inhibitory effect of anti-estrogens as some studies suggest that when ErbB2 and ER are co-expressed, patients respond poorly to endocrine therapy (Carlomagno *et al.*, 1996; Giai *et al.*, 1994; Houston *et al.*, 1999).

Estrogen represses ErbB2 expression and vice versa

Numerous studies also indicate that a mutually repressive feedback signaling loop exists between ErbB2 expression and that of the ER, probably reflecting the interrelationship of endocrine and paracrine signals important in normal mammary gland development as well as in cancer. Neuregulin administered to several breast cancer cell lines results in repression of ER transcription, although prior to this, a transient upregulation of ER activity has also been observed (Grunt *et al.*, 1995; Matsuda *et al.*, 1993; Mueller *et al.*, 1995; Pietras *et al.*, 1995; Tang *et al.*, 1996). Estrogen administration to breast cancer cell lines results in transcriptional repression of ErbB2. Independent studies found the ErbB2 promoter to be suppressed by either estrogen-induced downregulation or sequestration of the AP-2 or SRC-1 transcription factors respectively (Newman *et al.*, 2000; Perissi *et al.*, 2000).

The Yin and Yang association of ErbB2 to ER mirrors that for ErbB1 and the progesterone receptor (PR), where a dampening feedback loop was uncovered. Progestin administration induced a marked potentiation of EGF/Neuregulin induced signaling in T47D cells, as well as a significant upregulation of ErbB1, ErbB2 and ErbB3 protein levels (Lange *et al.*, 1998). However the progesterone-primed upregulation of ErbB signaling is probably short-lived as PR expression levels were also reported to collapse in cells exposed to EGF for longer periods (Lange *et al.*, 2000). These combined data thus suggest a functional negative interrelationship between steroid signaling and signaling through ErbB2 or ErbB1, and reflect alternate programs activated both during development and in mammary carcinogenesis.

In summary, the inverse relationship between ErbB1 or ErbB2 and steroid receptors may reflect different molecular programs exploited in the genesis of distinct breast cancer subtypes. Nevertheless, even though less common, ER-positive/ErbB2-positive and the reciprocal double-negative breast carcinomas do exist (Sjogren *et al.*, 1998), providing a stern reminder that the negative association between these markers is not absolute.

ErbB2 overexpression hotwires the cell cycle

The cyclin D connection

The D-type cyclins (D1, D2 and D3), which activate their catalytic partners CDK4 and CDK6, are induced by numerous mitogenic stimuli and play a central role in the kick-starting of the cell cycle. Aberrant overexpression of D-type cyclins can reduce or overcome the dependency of mitogenic stimulation for a cell, and thus can play a role in the process of oncogenic transformation (reviewed in Bartkova *et al.*, 1997; Sherr and Roberts, 1999; Weinberg, 1995). Therefore, it may not seem surprising that cyclin D1 plays a pivotal role in breast cancer, being overexpressed at a 40% incidence (Bartkova *et al.*, 1994). Its overexpression is particularly enriched (>75%) in ductal carcinomas *in situ* (DCIS) (Weinstat-Saslow *et al.*, 1995; Worsley *et al.*, 1997); the majority of which also overexpress ErbB2 (Allred *et al.*, 1992; Mack *et al.*, 1997). Distinct from overexpression, gene amplification of cyclin D1 occurs to a lesser extent, with greater frequency found in both DCIS and invasive lobular carcinoma. Other cyclin genes are rarely, if at all amplified in breast cancer (Bartkova *et al.*, 1994; Courjal *et al.*, 1996; Gillett *et al.*, 1994). These data implicate a generic requirement for cyclin D1 overexpression in different breast cancer subtypes, including those in which ErbB2 plays a role. In line with this notion, one of the two major phenotypes detected in cyclin-D1 knockout mice is a failure of alveolar lobule cells to expand during pregnancy, demonstrating a critical role for cyclin D1 during this period of rapid cell growth (Fantl *et al.*, 1995; Sicinski *et al.*, 1995).

Both estrogen-dependent and ErbB-dependent mitogenic signaling have been shown to channel through the activation of cyclin D1. Using a panel of cell lines transfected with either wild type or oncogenic ErbB2 (NeuT; Val^{664} to Glu), a dramatic upregulation of cyclin D1 protein expression has been demonstrated (Lee *et al.*, 2000). ErbB2-dependent signaling through cyclins D2 and D3 can also take place (Lane *et al.*, 2000; Neve *et al.*, 2000). This upregulation is at least in part initiated at the transcription level, and involves the SP1 and E2F transcription factors (Lee *et al.*, 2000). ErbB-dependent upregulation of the SP1 transcription factor has been independently reported (Alroy *et al.*, 1999). Cyclin D1 can be upregulated by activated Ras, Raf, MEK and Rac, all downstream targets of ErbB-signaling cascades (Albanese *et al.*, 1995; Aziz *et al.*, 1999; Cheng *et al.*, 1998; Gartel *et al.*, 2000; Gjoerup *et al.*, 1998).

Post-translational stabilization of the normally labile cyclin D1 is also an important process, and can be conferred by its threonine-phosphorylation by PKB/AKT (Diehl *et al.*, 1998). AKT is a major target of ErbB-signaling, specifically found downstream of ErbB3 or ErbB4 and perhaps also ErbB1 signaling complexes, via the activation of PI3 kinase (Elenius *et al.*, 1999; Liu *et al.*, 1999; Moscatello *et al.*, 1998; Prigent and Gullick, 1994; Waterman *et al.*, 1999). AKT activity was down-regulated in cell lines immunodepleted for ErbB2, thus providing an indirect post-transcriptional link between ErbB expression, PKB activation and cyclin D levels (Lane *et al.*, 2000; Liu *et al.*, 1999; Neve *et al.*, 2000). Taken together, these lines of evidence indicate that D-type cyclins are major down-stream targets of ErbB-dependent signaling, with upregulation possibly taking place both at transcriptional and post-transcriptional levels. A model of ErbB-induced tumorigenicity can thus be construed: ErbB2 amplification results in hyper-activation of a signaling network, which in turn dysregulates the G1/S checkpoint by the formation of high levels of active cyclin D-CDK4/6 complexes.

ErbB2 overexpression confers chemoresistance

Cancer cells overexpressing ErbB2 are often resistant to an array of cytotoxic agents and radiation damage (O'Rourke *et al.*, 1998; Tsai *et al.*, 1993; Yu *et al.*, 1996, 1998b). A mechanistic understanding of how ErbB2 confers chemoresistance remains somewhat elusive. However data to support this claim at the phenomenological level are abundant, and hints as to how ErbB2 overexpressing breast tumors evade not just cell cycle control, but also apoptosis, are beginning to surface.

The first direct evidence supporting that a central mediator of ErbB2's anti-apoptotic machinery may be the CDK inhibitor $p21^{Waf1}$ (Waf1, Cip1, Sdil) was published 2 years ago (Yu *et al.*, 1998a). This study examined the ErbB2-mediated protective role against paclitaxel (Taxol), normally a highly effective anti-neoplastic agent which induces apoptosis by interfering with the cell's microtubule machinery (Horwitz, 1992), but of limited therapeutic capacity in ErbB2-expressing tumors. Taxol-treated ErbB2 transfected MDA-MB-435 cells progressed less effectively towards the G2/M phase than control untransfected cells, with a decreased activation of cyclinB-Cdc2 and inhibition of apoptosis (Yu *et al.*, 1998a). In conjunction, the CDK inhibitor $p21^{waf1}$, was sharply upregulated in ErbB2-overexpressing cells, a phenomenon independently found for a

number of cell-lines treated with different ErbB-ligands (Bacus *et al.*, 1996b; Fan *et al.*, 1995, 1997; Fiddes *et al.*, 1998). To test if $p21^{Waf1}$ was a key mediator of the phenotypic differences observed between ErbB2 high and ErbB2 low expression, the effects of ErbB2 transfected into $p21^{Waf1-/-}$ knockout fibroblasts was examined. Overexpression of ErbB2 in wild-type fibroblasts conferred resistance to taxol-induced cell death, whereas protection against taxol toxicity was abrogated in ErbB2 overexpressing $p21^{-/-}$ cells (Yu *et al.*, 1998a). Thus these data indicate that elevated $p21^{Waf1}$ levels conferred by ErbB-signaling, elicits changes in the G2/M transition, and can help a cell escape from taxol-mediated cell death.

There are many roads to the upregulation of $p21^{waf1}$ (reviewed in Gartel and Tyner, 1999). Waf1 is a downstream target of the p53 tumor suppressor, and has been attributed a major role in p53-dependent cell cycle arrest (Deng *et al.*, 1995; el-Deiry *et al.*, 1993). $p21^{Waf1}$ is also directly upregulated through STAT transcription factors and also indirectly via the Ras-Raf-MAP kinase pathway, both downstream mediators of ErbB signaling (Gartel *et al.*, 2000; Kivinen *et al.*, 1999; Nosaka *et al.*, 1999). Indeed, $p21^{Waf1}$ was demonstrated to be upregulated in response to NRG1 in different cell types (Bacus *et al.*, 1996b). However, the sharp ErbB2-dependent activation of p21 and resistance to chemotherapy takes place in cells even in the absence of functional p53 (O'Connor *et al.*, 1997; Yu *et al.*, 1998a). Furthermore, depletion of ErbB2 expression results in a subsequent decrease in $p21^{Waf1}$ levels but not p53 levels (Pietras *et al.*, 1999). These combined data indicate a p53-independent role of $p21^{Waf1}$ in breast tumors.

Why p21, a CDK inhibitor, may enhance tumorigenicity

$p21^{waf1}$ was originally identified as a universal CDK inhibitor. It was reported to bind to and inhibit all cyclin/CDK complexes of the cell cycle, with the capacity to induce cell growth arrest (Xiong *et al.*, 1993). Thus it may seem rather perplexing that p21 plays a major role in ErbB2-dependent oncogenicity. However, upon closer inspection, the role of p21 in the cell is far more complex. p21 as well as other CDK 'inhibitors' can bind with different stoichiometric ratios to higher order cyclin-CDK complexes. Lower order complexes with a 1:1:1 subunit ratio of cyclin:CDK:$p21^{Waf1}$ are thought to confer an active state, whereas additionally bound p21 subunits can at least in some cases, elicit an inhibitory effect (Zhang *et al.*, 1994). Low concentrations of $p21^{waf1}$ and another CDK inhibitor, $p27^{Kip1}$, were even demonstrated to enhance the kinetics of assembly of the CDK4/cyclin D complex by more than an order of magnitude over complexes that lacked these 'inhibitors'. In fact, in primary fibroblasts lacking both $p21^{Waf1}$ and $p27^{Kip1}$, cyclin D-CDK complexes failed to form at all (Cheng *et al.*, 1999; LaBaer *et al.*, 1997). Thus members of the p21 CDK family more correctly play activating or inhibitory roles, dependent on their relative expression levels in respect to their corresponding cyclin/CDK cell partners.

Under physiological conditions, it seems that $p21^{Waf1}$ and $p27^{Kip1}$, which can act as inhibitors of cyclin E- and A-dependent kinases, also act to positively regulate cyclin D-dependent kinases (Cheng *et al.*, 1999; Polyak *et al.*, 1994; Toyoshima and Hunter, 1994). A major non-catalytic function of cyclin D-dependent kinases is to bind p21 and p27, thus releasing their repressive constraint of these other components of the cell cycle machinery (Geng *et al.*, 1999; Planas-Silva and Weinberg, 1997). Therefore, ErbB2 overexpression in breast cancer cells results in a striking upregulation of cyclin D1-CDK activity, an activity which is not necessarily perturbed by upregulated $p21^{waf1}$ levels. Interestingly, the literature presents conflicting reports that Neuregulins (NRGs) can induce either proliferation, differentiation or apoptosis in different breast cancer cell-lines (Aguilar *et al.*, 1999; Daly *et al.*, 1997; Guerra-Vladusic *et al.*, 1999; Weinstein *et al.*, 1998). We propose a model in which the fate of a cell in response to ErbB-ligand stimulation is a co-function of the amplitude of ErbB-activation, p53 status and the cyclin D-p21/p27 ratio, where the switch from NRG-dependent differentiation or apoptosis to proliferation is conferred by hyper-activation of the ErbB signaling network (Figure 3).

Cancer-inhibitory anti-ErbB2 antibodies

The cancer-inhibitory potential of antibodies to ErbB2 was realized as far back as 1984, when mice bearing tumors with an active form of the rodent ErbB2 were treated with specific antibodies (Drebin *et al.*, 1984). However, only within the last 4 years has this realization yielded a method to treat cancer patients (Baselga *et al.*, 1996). Although antibodies conjugated to toxins, radionucleotides and prodrugs show promising results in animal models, it is important to note that even naked forms of antibodies to ErbB2 are active in arresting tumorigenic growth of ErbB2-overexpressing cells. Relevant to the subject of this review is the exact mechanism by which anti-ErbB2 antibodies inhibit cell growth. Experiments performed on various systems suggest that these antibodies can induce differentiation of rapidly dividing breast cancer cells to growth-arrested milk-producing cells (Bacus *et al.*, 1992). Likewise, it has been reported that anti-ErbB2 antibodies down-regulate expression of angiogenic growth factors in tumor cells, both *in vitro* and in animal models (Petit *et al.*, 1997). Furthermore, ErbB2-mediated resistance to the cytotoxic effects of the tumor necrosis factor can be significantly reduced by monoclonal antibodies to ErbB2 (Hudziak *et al.*, 1988).

Mechanistically, the antibodies appear to act by removing ErbB2 from the cell surface. It has previously been shown that the oncogenic activity of ErbB2 necessitates its localization at the plasma membrane (Flanagan and Leder, 1988). In line with blocking ErbB2 action by preventing its oncogenic interaction with other surface-localized ErbB proteins, the tumor-inhibitory potential of specific antibodies (Hurwitz *et al.*, 1995) or certain combinations of anti-ErbB2 antibodies (Kasprzyk *et al.*, 1992) correlates with the efficiency of antibody-induced down-regulation of ErbB2. Consistent with this notion is the observation that antibody bivalence is essential for inhibitory activity. However, effects mediated by the Fc portion of these antibodies also contribute to their tumor-arresting activity (Clynes *et al.*, 2000). Because all

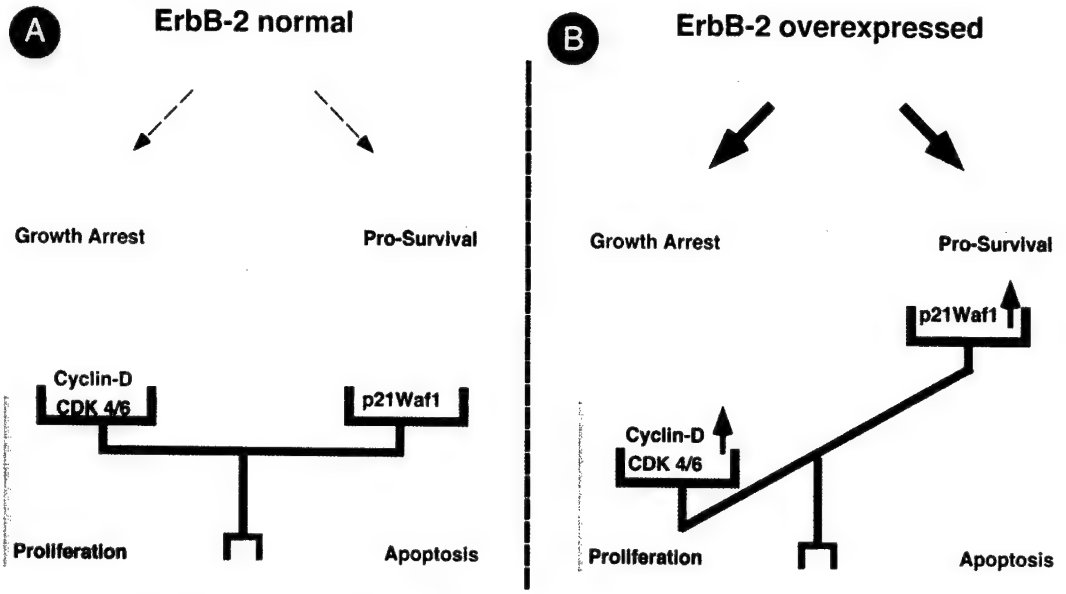

Figure 3 Apoptosis, differentiation or proliferation induced by the ErbB-signaling network. A model is proposed in which three key factors contribute towards the phenotypic response to ErbB-signaling: Amplitude of ErbB-signaling as well as levels of both D-type cyclins and $p21^{Waf1}$. (**a**) With limiting ligand concentrations or physiological levels of cell surface receptors, signaling through the ErbB network can induce apoptosis, differentiation or cell division, in a delicate balance dictated by cyclin D activation and levels of the cell cycle inhibitor $p21^{Waf1}$, as well as p53. Other CDK inhibitors, such as $p27^{Kip1}$ also play a role in the differentiation process (not shown). (**b**) Amplification of ErbB2 results in hyper-activated signaling of the ErbB signaling network which in turn sharply upregulates cyclin D1 and $p21^{Waf1}$, resulting in formation of active higher order cyclin D-CDK complexes and subsequent release from the G1/S checkpoint. Some evidence even indicates a pro-survival activity conferred by $p21^{Waf1}$ overexpression, although the mechanism in which this takes place in the context of breast carcinoma has not been elucidated

bivalent antibodies to ErbB2 share partial agonistic activity, their ability to down-regulate surface ErbB2 may relate to kinase activation. Indeed, recent evidence suggests that autophosphorylation of ErbB2 and subsequent recruitment of c-Cbl are necessary for antibody-induced down-regulation of ErbB2 (Klapper *et al.*, 2000b).

As a consequence of down-regulation, antibodies to ErbB2 elevate $p27^{Kip1}$ and the Rb-related protein p130, resulting in a reduction in the number of cells in the S phase of the cell cycle (Sliwkowski *et al.*, 1999). In independent studies, ErbB2-inhibitory antibodies curtailed the levels of $p21^{Waf1}$ in cells overexpressing ErbB2, although levels of cyclin D1 remained relatively unchanged (Pietras *et al.*, 1998, 1999; Ye *et al.*, 1999). When examining all known cyclin D members, it was found that cyclins D2 and D3 decreased dramatically (Lane *et al.*, 2000; Neve *et al.*, 2000). Thus the downstream effects of blocking antibodies to ErbB2 translate to signals that inhibit progression through the cell cycle and can explain their clinical benefit.

Future perspectives

Our current understanding of the biological role of ErbB proteins in normal physiology and in pathological states is due to the remarkable convergence of experimental data. Molecular cloning of the major players, their studies *in vitro* and subsequent mutagenesis in model animal systems paved the way to the notion that growth factors and their ErbB surface receptors function in the framework of a communication network. Studies in invertebrates not only helped in tracking the evolution of the network, but also provided genuine physiological perspectives and highlighted the backbone of the primordial network.

In-depth understanding is certainly the key for rational design of therapeutic strategies aimed at the ErbB network. Cancer therapy has taken center stage with the development of tumor-inhibitory antibodies to ErbB1 and ErbB2. Along these lines, novel drugs that inhibit tyrosine kinase activity, interfere with ligand binding, or block transcription of specific components of the network, are expected to reach maturation within the next decade. Some of the pharmacological agents may be useful in the treatments of wound, psoriasis and other hyperproliferative diseases in which the ErbB network is involved.

Despite the overwhelming linkage between basic research and clinical applications, serious gaps in our understanding still exist. For example, we still lack the conceptual framework for cell fate determination. This is best exemplified by the heterogeneity of epithelial organs such as the mammary gland. Construction of a hierarchy of cell lineages, analogous to hematopoiesis, will greatly impact upon the diagnostic and prognostic value of ErbB2, its ligands and their relation to the steroid hormone axis. Such understanding is expected to emerge from studies in animal systems, especially inducible gene targeting and knock-in of mutated components of the ErbB network. On the other extreme, we lack a high resolution structural understanding of the interactions between growth factors and their ErbB receptors. Three dimensional resolution of receptor dimers, especially those containing ErbB2,

will allow design of specific antagonists. This promising but currently non-existing avenue awaits successful crystallization of ErbB-ligand complexes.

Lastly, the life of an epithelial or any other type of cell, is controlled by more than one signaling network. Resolution of the interaction between ErbB proteins and G-protein coupled receptors, cytokine receptors, cell adhesion molecules and other networks poses a new challenge to basic researchers. How a convergence of networks is integrated in the cytoplasm and impacts transcriptional programs is still a darkened area. Shedding light on the way information is integrated and translates into specific outputs, such as cell cycle progression, is not just a theoretical issue. For example, the question why specific combinations of anti-ErbB2 antibodies and chemotherapeutic drugs are cardiotoxic, may have not remained an enigma if we understood how the ErbB network interacts with the apoptotic pathway. Undoubtedly more questions will arise from the interface between clinical research and basic research, where we will face more complex challenges.

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Minireview

Molecular mechanisms underlying endocytosis and sorting of ErbB receptor tyrosine kinases

Hadassa Waterman, Yosef Yarden*

Department of Biological Regulation, the Weizmann Institute of Science, Rehovot 76100, Israel

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Abstract The major process that regulates the amplitude and kinetics of signal transduction by tyrosine kinase receptors is endocytic removal of active ligand–receptor complexes from the cell surface, and their subsequent sorting to degradation or to recycling. Using the ErbB family of receptor tyrosine kinases we exemplify the diversity of the down regulation process, and concentrate on two sorting steps whose molecular details are emerging. These are the Eps15-mediated sorting to clathrin-coated regions of the plasma membrane and the c-Cbl-mediated targeting of receptors to lysosomal degradation. Like in yeast cells, sorting involves not only protein phosphorylation but also conjugation of ubiquitin molecules. The involvement of other molecules is reviewed and recent observations that challenge the negative regulatory role of endocytosis are described. Finally, we discuss the relevance of receptor down regulation to cancer therapy.

Key words: Clathrin; Growth factor; Oncogene; Review; Signal transduction; Tyrosine kinase; Ubiquitin

1. Introduction

Exposure of cells or tissues to a variety of hormones and growth factors almost invariably leads to disappearance of specific binding sites from the cell surface. This phenomenon, which is due to accelerated endocytosis of ligand–receptor complexes, was termed 'down regulation'. Down regulation of one group of receptor tyrosine kinases (RTKs), those that bind growth factors sharing an epidermal growth factor-(EGF-) like motif, is one of the most extensively analyzed. The four members of this group, ErbB-1 through ErbB-4, are transmembrane proteins whose large extracellular domains bind specific growth factors, whereas their intracellular domains are endowed with tyrosine kinase activity (reviewed in [1]). The monomeric forms of ErbB proteins are catalytically much less active than the ligand-induced dimers, whose autophosphorylation recruits diverse phosphotyrosine binding proteins to initiate signal transduction. Because the four ErbB proteins can form both homo- and heterodimers, and each receptor can recruit a specific set of signalling proteins, this configuration allows enormous potential for signal diversification. Moreover, one ErbB protein, namely ErbB-3 is devoid of enzymatic activity [2], whereas ErbB-2 seems to function solely as a low affinity co-receptor [3]. Thus, signalling by ErbB receptors and their many ligands may be considered in terms of a layered network whose output depends on combinatorial interactions.

Uniquely, the ErbB network can be tracked in evolution to a primordial simple module in worms. The single ErbB ortholog of *Caenorhabditis elegans* is activated by only one ligand, called Lin3, and it transmits signals primarily through the Ras pathway. This linear pathway evolved throughout evolution to form a richly interconnected network, whose complexity derives from the existence of many ligands and four receptors capable of forming 10 dimeric combinations. Although signalling down-stream of all four mammalian ErbB proteins is funneled into the Ras pathway, variation exists in terms of the specific repertoires of phosphotyrosine binding proteins that are recruited to each receptor. Moreover, the various dimeric receptors differ in the potency of mitogenic signals, presumably because each ErbB protein follows a unique pathway of endocytosis and down regulation. For example, ErbB-1 is rapidly internalized and degraded following activation by some ligands, but internalization of ErbB-2 and the two neuregulin receptors, ErbB-3 and ErbB-4, is relatively slow [4,5]. Because ErbB-3 is devoid of enzymatic activity and this function is essential for degradation of internalized receptors [6], this neuregulin receptor recycles back to the plasma membrane, perhaps after unloading its ligand in an endosomal compartment [7].

A wealth of experimental evidence has established the notion that the kinetics of signalling by ligand-activated receptors determines not only the amplitude of the output but also its specificity (e.g., mitogenesis or differentiation) [8]. Consistent with this paradigm, a mutant ErbB-1 whose endocytosis is impaired can deliver oncogenic signals [9], and several oncogenic animal viruses impair endocytic removal of active ErbBs from the cell surface. Examples include the E5 protein of human papilloma virus, which blocks an endosomal ATPase, thus shunting internalized receptors to the recycling pathway [10]. Poxviruses encode multiple EGF-like ligands that bind with relatively low affinity to ErbB proteins. However, because the viral ligands cause only limited receptor down regulation, their mitogenic potency is enhanced relative to the mammalian counterpart [11]. Retroviruses present a variety of mechanisms that help them evade receptor down regulation: Oncogenic Ras proteins appear to slow down the rate

*Corresponding author. Fax: (972)-8-9342488.
E-mail: yosef.yarden@weizmann.ac.il

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of internalization, whereas the oncogenic v-ErbB receptor encoded by the avian erythroblastosis virus is devoid of a phosphorylation site essential for targeting to lysosomal degradation [12].

Despite their importance, negative regulatory processes are less understood than the steps involved in signal generation and propagation to the nucleus. Lessons derived from the endocytic pathways followed by cargo receptors like the transferrin receptor (TfR) and the low density lipoprotein (LDL) receptor are only partially relevant to signalling receptors like ErbBs. Nevertheless, the list of proteins that participate in receptor inactivation is steadily increasing and we now begin to understand their interactions along the endocytic routes. After dealing with endocytosis in simple eukaryotes, namely yeast cells, we concentrate on two sorting processes that determine receptor down regulation. These are sorting of receptors to internalization through the clathrin-coated pit and their later interaction with a machinery that determines lysosomal degradation.

2. Lessons from yeast

Genes that are not essential for viability can be deleted from the yeast genome and thus enable direct examination of their cellular role. The usefulness of this approach has been repeatedly exemplified by the isolation of mutant yeast cells defective in certain steps of endocytosis. Recent ultrastructural and biochemical approaches suggest that the general organization of endocytic traffic inside yeast cells resembles that of mammalian cells. Both early and late endosomes have been morphologically and biochemically identified in yeast [13,14], and the molecular machinery required for vesicular transport in yeast does not seem to be fundamentally different from the mammalian machinery [15].

Due to the lack of refined assays to investigate intracellular post-endocytic steps, this phase is less understood than the internalization step, which has been thoroughly investigated in yeast. Interestingly, most yeast transmembrane proteins, even transporters specific for certain metabolites [15,16], seem to undergo constitutive endocytosis. However, some of these endocytic systems display a large increase in the rate of endocytosis upon varying growth conditions or, in the case of pheromone receptors, by the addition of a ligand to the growth medium. Several genes that are potentially essential for accelerated endocytosis were identified. Consequently, some striking similarities and differences between yeast and mammalian internalization became apparent. One such protein is the clathrin heavy chain (CHC), a main component of the clathrin coat and a major player in membrane sorting. Surprisingly, inactivation of the gene (CHC1), using a temperature sensitive allele, resulted in moderate (50%) reduction in the rate of a-factor internalization [17]. This partial effect of a clathrin mutation on endocytosis in yeast may be explained by the existence of two parallel internalization pathways: a clathrin-dependent route as well as a clathrin-independent pathway, as is the case in mammalian cells.

Another aspect relates to the classical endocytic signals identified in mammalian plasma membrane proteins (e.g. YXXΦ, where Φ is a bulky hydrophobic amino acid, MPXY, and a di-leucine motif). These motifs bind specific subunits of the AP-2 adapter complex of clathrin, but only rarely have similar signals been identified in yeast. One example is the linear sequence NPFTD, which is required for ligand- and clathrin-dependent endocytosis of the a-factor receptor [18]. Similarly, the di-leucine motif is required for endocytosis of the Gap1p permease [19]. In neither case have the interacting molecules been identified. However, in several other cases the endocytic signals identified in yeast correspond to motifs required for protein ubiquitination (reviewed in [20]). The identified ubiquitination signals are relatively long acidic sequences. At least in the case of Ste2p, Ste3p and Fur4p, the acidic nature seems to be modulated by phosphorylation [21–23]. A phosphorylation-based recognition signal for endocytosis is an appealing possibility in both yeast and mammals, because ubiquitination and/or endocytosis of many receptors and transporters are often regulated by ligands, substrates or extracellular nutrients.

Similar to clathrin, the involvement of dynamin in endocytosis has been extensively documented in animal cells, but its role in yeast is rather limited. Although three dynamin homologs were identified in yeast, Mgm1p, Vps1p and Dnm1p, none is absolutely required for internalization of membrane proteins [24,25]. By contrast, Pan1p, the yeast homolog of the mammalian EGF-receptor protein substrate Eps15, is essential for normal endocytosis. The polyvalent structure of Pan1p implicates it as a key regulator that may transiently recruit components of the endocytic machinery [26]. An interesting genetic interaction between Pan1p and the ubiquitin ligase Rsp5p (the yeast homolog of human Nedd4) has been identified [27]. This WW and HECT domain protein is required for endocytosis of many yeast proteins [27–29], and although its biochemical interaction with Pan1p is still uncharacterized, lessons accumulating with Nedd4 raise the possibility that Pan1p acts as an adapter connecting the ligase to potential ubiquitination substrates. That Pan1p may itself be ubiquitinated by Rsp5p is suggested by the observation that Eps15 is ubiquitinated in mammalian cells [30].

3. Major endocytic routes of membrane receptors

Several morphologically and functionally different types of endocytic pathways exist in animal cells. Phagocytosis represents the uptake of solid particles (>0.5 μ diameter) that must bind to specific plasma membrane receptors capable of triggering their own endocytosis, usually by causing the formation of F-actin-driven pseudopods that envelop the bound particle. On the other hand, pinocytosis enables uptake of extracellular fluid, macromolecules and solutes bound specifically or non-specifically to the plasma membrane. A third pathway, receptor-mediated endocytosis, is the selective adsorptive uptake of specific macromolecules bound to plasma membrane receptors (see Fig. 1). This process involves constitutive formation of small (<0.2 μ diameter) vesicles, which is usually preceded by the formation of clathrin-coated vesicles (CCVs). The basic components of the clathrin coat have long been known [31,32]. A tri-skelion of three CHCs tightly associated with three clathrin light chains constitutes the assembly unit of the polygonal lattice. A hetero-tetramer (AP-2) consisting of two large (α and β) and two small subunits (σ2 and μ2) mediates assembly of clathrin cages on the plasma membrane. The β subunit interacts with clathrin [33], whereas the μ2 subunit is capable of binding the tyrosine-based endocytic signals that mediate internalization of a number of membrane proteins [34,35].

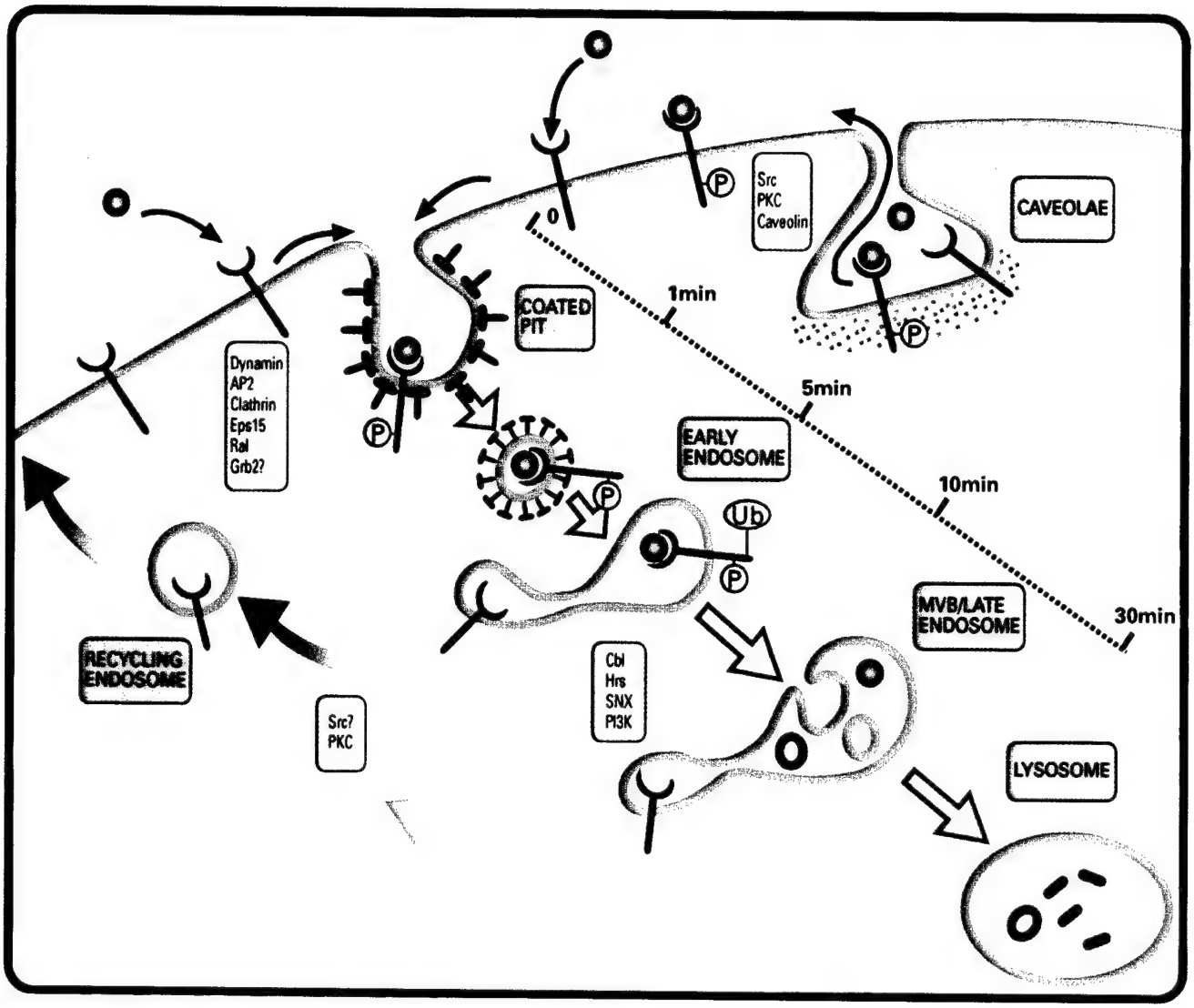

Fig. 1. Model of endocytosis of RTKs based on the trafficking of ErbB-1. The major route of endocytosis from the clathrin-coated pit are marked and the time scale indicated. Unoccupied receptors may be concentrated in caveolae and in non-caveolar regions of the cell surface, but upon ligand binding and activation of the intrinsic kinase, caveolar receptors translocate out of the caveolae. The potential major players of caveolar retention and exit are indicated. The mechanism that allows recruitment of the AP2 complex and a clathrin coat are regulated by a large number of adapters and enzymes, including dynamin and Eps15. Following formation of a clathrin-coated vesicle and its uncoating to form the early endosome, the second major sorting event takes place. This involves sequestration of active receptors in inner vesicular structure of the multi vesicular body. On the other hand, receptors whose ligands are released due to the acidic pH of the endosomal compartment, are directed to tubular structures. The sorting process appears to involve recruitment of c-Cbl, receptor ubiquitination and inward vesicular transport regulated by lipid kinases. Recycling of receptors back to the plasma membrane occurs from most endosomal compartments, but its efficiency is gradually reduced, as the compartment becomes deeper in the cytoplasm. Recycling from the tubular portion of the MVB may involve specific protein kinases such as PKC and Src.

An alternative to clathrin-mediated endocytosis involves specialized forms of rafts, glycosphingolipid and cholesterol-enriched microdomains, termed caveolae. These are small invaginations that exist on the surface of many cell types. The flask-shaped caveolar pit is characteristically 50–80 nm in diameter, highly uniform, and enriched in caveolins, sphingolipids and cholesterol [36–38]. Caveolae are coated with a spiral-shaped striated coat, which is structurally different from clathrin lattices. Molecules internalized through caveolae (reviewed in [38]) may travel to the cytoplasm or to the endoplasmic reticulum. Alternatively, they may be directed to a caveolae derived tubular/vesicular compartment. Caveolae and clathrin-coated pits are specialized to internalize different types of molecules. Therefore, caveolae-mediated, and clathrin-mediated endocytosis are parallel, but non-overlapping, endocytic pathways. Moreover, although several proteins implicated in vesicle trafficking have been localized to caveolar fractions, it is unclear whether tubular or vesicular caveolae ever fuse with endosomes originating in coated pits [39].

4. Receptor sorting to the clathrin-coated vesicle

4.1. Exit from caveolae

In quiescent fibroblasts a relatively large fraction of ErbB-1 is concentrated in caveolae [40–42], but other reports suggested that most of the receptor of overexpressing cells is

confined to the low-buoyant density fraction, representing non-caveolar membrane domains [43]. Structural analysis revealed that the information required for delivery of ErbB-1 to caveolae is contained within the transmembrane and juxtamembrane domains of the receptor, distinct from a caveolin-1 binding domain [44]. In response to EGF, the total number of surface receptors decreases, along with a decline in the percentage of EGF receptors in the caveolar fraction. Depending on the cell type, it takes 3–30 min for ErbB-1 to leave caveolae [41]. The rapid exit appears to require autophosphorylation of at least one of the five major tyrosine residues in the regulatory domain of the receptor, as well as an intact kinase activity [45]. In addition to ligand binding, Src family kinases may control receptor traffic out of caveolae, as a synthetic Src inhibitor can inhibit receptor exit. Consistent with this model, overexpression of Src stimulates an increase in the rate of receptor endocytosis [46]. On the other hand, movement out of caveolae is inhibited by activators of protein kinase C (PKC). Interestingly, overexpression and truncation of ErbB-1 cause a marked phosphorylation of caveolin-1, a major component of caveolae whose exact function is still unclear [47,48]. Noteworthy is the fact that migration from caveolae is uncoupled to internalization through clathrin-coated pits, as blocking clathrin mediated endocytosis does not affect ligand-stimulated depletion of the receptor from the caveolar fraction.

4.2. Interactions with the AP2 recruiting complex

Natural mutations of the LDL receptor helped uncover the determinants needed for recruitment of constitutively internalizing receptors to the clathrin/AP-2 complex [49], and also involved a particular tyrosine residue [50]. Since then, a large variety of internalization signals have been identified by site-directed mutagenesis of various cell-surface proteins. Although structurally heterogeneous, these signals may be divided into two groups (reviewed in [35,51]). The first group is characterized by an essential tyrosine, which is part of the motif NPXY or YXXΦ. Resolution of the crystal structure of an internalization signal (a YXXΦ peptide) bound to the μ2 subunit of AP2 showed that the peptide assumed an extended conformation, and specificity was conferred by hydrophobic pockets that bound the tyrosine and the hydrophobic residues of the peptide [52]. A second group of internalization motifs typically contains a di-leucine sequence, but in some cases one of the leucines may be replaced by an isoleucine, valine or an alanine. In the case of ErbB-1, a stoichiometric complex with AP2 has been attributed to an internalization signal flanked by tyrosine residue 974 [53]. However, mutant receptors lacking the putative AP2 binding site can undergo internalization via clathrin-coated pits [54]. Likewise, the two di-leucine motifs of ErbB-1 may not play a role in internalization of a full-length receptor [55–58]. Presumably, internalization signals allow only low affinity interactions between ErbB proteins and AP2. This may be sufficient for slow constitutive internalization of unoccupied or kinase-defective receptors, but additional interactions may be involved in ligand-stimulated recruitment into coated pits. In line with this scenario, the rapid endocytic pathway of ErbB-1 is saturable [59], but saturation of the endocytic pathway for TFRs does not affect endocytosis of ErbB-1 [60].

4.3. Proteins involved in ligand-regulated recruitment

The three major components of the coated pit are clathrin, AP2 and dynamin. AP-2 drives clathrin assembly and recruits the cytoplasmic tails of constitutively internalizing receptors, as well as ligand-induced receptors. On the other hand, clathrin defines the structure of the pit and dynamin is responsible for fission of the vesicle from the plasma membrane. This large GTPase is thought to act as a mechano-enzyme that mediates the constriction (liberation) of nascent clathrin-coated pits from the plasma membrane during endocytosis [61]. In vitro studies indicate that dynamin binds to membrane-embedded phosphoinositides via its PH domain, and its GTPase activity constructs and fragments membrane tubules capped by clathrin-coated buds [62] (see Fig. 2). A mutant dynamin defective in the GTPase activity blocks ligand-induced endocytosis of ErbB-1, but constitutive internalization is not affected [63]. Unlike constitutively internalizing receptors, which directly interact with AP2, the endocytic signals of ligand-induced receptors may be exposed only upon receptor autophosphorylation. Indeed, kinase activity and a phosphorylation substrate are required for efficient recruitment of ErbB-1 but not TfR [64]. The identity of the substrate(s) remains unknown. However, recent work by Di-Fiore and colleagues implicated an AP2 binding protein, Eps15, in accelerated endocytosis of ErbB-1 [65], and analyses of signalling downstream to the Ral small GTPase attributed a role in endocytosis to another AP2 binding partner, namely RalBP1 [66,67].

4.3.1. Eps15 and Eps15R. These related proteins are endowed with multiple binding specificities: three copies of the EH domain bind to NPF motifs of NUMB and other proteins, while the centrally located coiled coil region allows homodimerization or heterodimerization with other coiled coil proteins such as intersectin. Finally, the COOH-terminal region, which is characterized by repeated DPF tripeptides binds the α subunit of the AP2 complex (reviewed in [68]). Upon activation of ErbB-1, Eps15 is recruited to the plasma membrane [69] and localizes to coated pits [70]. By electron microscopy, Eps15 was found to localize to the rim of the budding-coated vesicle and not to deeper invaginations. The rim is the growing part of the forming pit. During coat assembly the rim is the only site where Eps15 remains associated with AP-2, but once clathrin polymerization has taken place, Eps15 may be excluded from clathrin/AP2 complexes [71]. Consistent with an essential role in receptor-mediated endocytosis, expression of dominant negative mutants of Eps15 or microinjection of neutralizing antibodies inhibited endocytosis of both ErbB-1 and TfR [72,73]. However, tyrosine phosphorylation of Eps15 is required exclusively in the process of ligand-induced receptor internalization. Thus, an Eps15 mutant defective in the major tyrosine phosphorylation site (tyrosine 850) specifically inhibited internalization of ErbB-1, but did not affect internalization of TfR [65]. Because the corresponding phosphopeptide can block internalization, it is conceivable that following ligand binding and elevated phosphorylation of tyrosine 850, an unknown phosphotyrosine binding protein binds to the modified tyrosine and selectively accelerates recruitment of occupied receptors to the AP2 complex. In conclusion, Eps15 may fulfil a dual role; while it is essential for endocytosis of constitutively internalizing receptors, its phos-

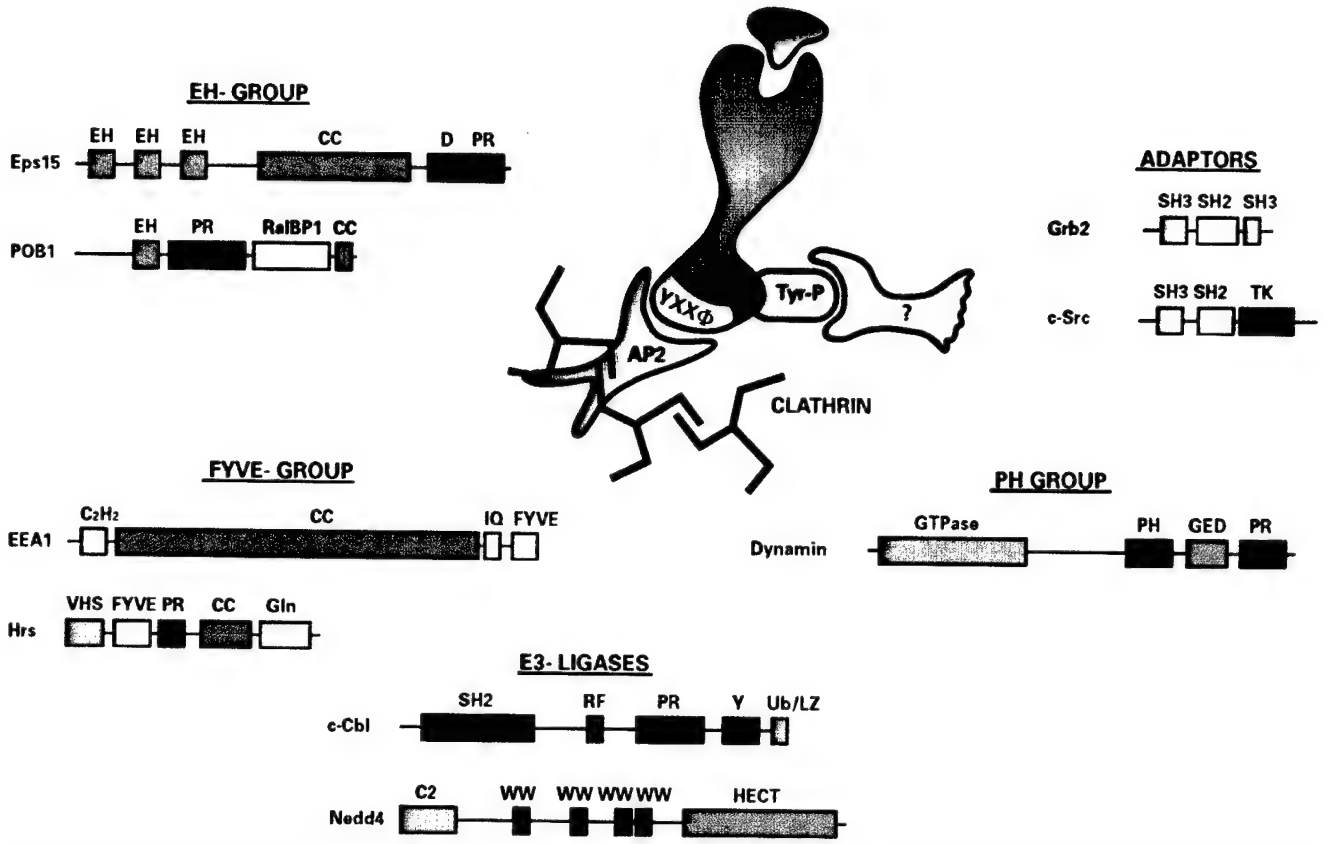

Fig. 2. Molecules involved in sorting RTKs along the endocytic pathway. The transmembrane receptor is schematically depicted in a complex with AP2 and a clathrin cage. The interaction of AP2 with the cytoplasmic part of the receptor is mediated by an endocytic signal such as the YXXΦ motif, but other adapters, whose engagement depends on tyrosine phosphorylation, may enhance recruitment of the AP2 complex. Molecules involved in this sorting event, as well as the sorting that occurs in multi vesicular bodies are grouped and schematically presented. The structural domains are shown in different colors and their names indicated. The abbreviations used are: CC, coiled coil, C2, a phospholipid and calcium binding region; D, a region containing DPF repeats; EH, Eps15 homology; FYVE, a PI3P binding; RF, a RING finger domain; PR, a proline-rich domain; SH, Src homology domain; TK, tyrosine kinase.

phorylation is required only for the rapid, ligand-induced endocytosis of ErbB-1.

4.3.2. RalBP1 and POB1. Ral is a member of the small GTP binding protein family [74,75]. The only known effector protein of Ral, RalBP1, and its own partner POB1, are both implicated in EGF signalling downstream of Ras [76]. Exposure of cells to EGF or to insulin increases the GTP-bound active form of Ral through activation of Ras and its effector, a guanine nucleotide exchange factor for Ral (RalGEF) [77–79]. Active Ral binds to the C-terminal part of RalBP1, a putative GTPase of Rac1 and CDC42 [75]. While the relevance of this GAP activity to endocytosis remains unknown, RalBP1 can effectively recruit the AP2 complex, either directly or through POB1 and Eps15 (see below). The μ2 chain of AP2, but not other coat proteins, binds to the N-terminus of RalBP1, and inhibition of these constitutive interactions blocks endocytosis of both ErbB-1 and TfR [67]. On the other hand, phosphorylation of POB1, a binding partner of RalBP1, Eps15 and epsin, another EH domain protein that participates in clathrin-mediated endocytosis [80], is elevated by EGF. Thus, recruitment of POB1 to the AP2 complex may be involved in the ligand-induced pathway. Indeed, deletion mutants of POB1 can inhibit endocytosis of both ErbB-1 and the insulin receptor [66]. Presumably, RalBP1 is translocated to the plasma membrane upon stimulation with EGF and subsequent activation of Ras and Ral. Once associated with the plasma membrane, RalBP1 can bind AP2 in a complex manner that involves not only constitutive and ligand-induced interactions, but also the intrinsic GTPase activity.

4.3.3. c-Src and Grb2. A role for c-Src in EGFR-mediated responses has been demonstrated by a number of studies showing that it is required for EGF-induced mitogenesis and tumorigenesis [81,82]. The mechanisms by which c-Src influences the biological action of ErbB-1 are diverse: c-Src may potentiate receptor activity by binding to the receptor and inducing its phosphorylation, resulting in enhanced downstream signalling [81]. Alternatively, there may be a mutual catalytic regulation of the receptor and c-Src [83]. However, accumulating results attribute to Src a role in the endocytic trafficking of RTKs. A significant fraction of c-Src in fibroblasts has been found associated with endosomes [84], and the SH3 domain of c-Src is capable of binding and activating dynamin [85]. Overexpression of Src leads to an increase in the rate of endocytosis of ErbB-1 [46]. The under-

lying mechanism was investigated in cells lacking endogenous Src family members, or in cells treated with the Src inhibitor PP1. These experiments showed that endocytosis of the activated receptor is delayed when Src activity is inhibited [86]. Furthermore, Src activation leads to tyrosine phosphorylation of the CHC at tyrosine 1477, located at a region involved in clathrin assembly. Consequently, clathrin undergoes redistribution to the cell periphery, which may explain how Src is involved in induced endocytosis [86]. The exact involvement of another target of RTKs, Grb2, is less understood. However, this SH3-SH2-SH3 adapter protein (see Fig. 2) binds to a large variety of cellular proteins, including some effectors of endocytosis such as POB1, amphiphysin and synaptojanin. In addition, the SH3 domains of Grb2 interact with dynamin to activate its GTPase in synergy with phosphoinositides [87]. That some of these interactions are necessary for endocytosis of ErbB-1 is indicated by the inhibitory effect of a microinjected fusion protein containing the SH2 domain of Grb2, or the corresponding phosphopeptide ligand [88].

5. Sorting in the multivesicular body (MVB)

Once sorted to clathrin-coated vesicles, internalized receptors are delivered within 2–5 min to a tubular–vesicular network located at the cell periphery (Fig. 1). After 10–15 min, ligand–receptor complexes accumulate in relatively large perinuclear vesicles that contain internal vesicles (MVBs) [89]. These intermediate endosomes are characterized by an accumulation of hydrolytic enzymes, and low internal pH, sufficiently acidic to dissociate some ligands. Studies performed with recycling receptors and kinase-defective mutants of ErbB-1 imply that the MVB is the major site of sorting to lysosomal degradation. Unlike TFRs and kinase-dead ErbB-1 molecules, which are confined to the vesicular portion of MVBs, internalized ErbB-1 molecules accumulate in the inner vesicles of the MVB [6,90]. It is thought that translocation of active ErbB-1 molecules from the perimeter of the MVB to internal vesicles requires phosphorylation of an endosomal substrate that allows, perhaps together with ancillary proteins, removal of the receptor from the recycling pathway. The mechanisms underlying regulation of this critical sorting event currently begin to surface with the identification of candidate molecular players we discuss below.

5.1. c-Cbl

Members of the Cbl family of adapter protein are early prominent substrates for tyrosine phosphorylation by activated receptors for growth factors, cytokines, and immunoglobulins (reviewed in [91]). A single Cbl ortholog, Sli1, exists in nematodes and genetic evidence attributed to it a major negative regulatory role downstream of Let23, the ErbB ortholog of worms [92]. c-Cbl consists of an N-terminal unique Src homology domain (SH2), which mediates binding to tyrosine-phosphorylated receptors [93], and a C-terminal half that carries a long proline-rich domain and several tyrosine and serine phosphorylation sites, serving in constitutive and inducible interactions. A centrally located ring-finger (RF) domain separates the two adapter domains. The RF is missing or defective in two oncogenic forms of c-Cbl, v-Cbl and 70Z-Cbl, suggesting a role in the negative function of c-Cbl. Interestingly, c-Cbl cannot interact with ErbB-3 and ErbB-4, two receptors whose ligand-induced down regulation is impaired [94]. Indeed, overexpression of c-Cbl enhances down regulation of ErbB-1 and also increases ligand-induced ubiquitination of this receptor [95]. Recently, c-Cbl was identified as a ubiquitin ligase whose RF recruits an ubiquitin-loaded E2 enzyme [12,96], thus establishing its direct role in ubiquitination of ErbB-1. However, the exact site of action of c-Cbl is a matter of controversy. Evidence derived from experiments with yeast (see above), the growth hormone receptor [97] and blocking ErbB-1 internalization with a dynamin mutant [98] suggested that ubiquitination may be associated with sorting at the plasma membrane. In addition, translocation of c-Cbl to the plasma membrane was observed in macrophages [99]. On the other hand, several groups reported on the endosomal localization of c-Cbl and its co-localization with internalized receptors [95,100,101]. Moreover, the phosphorylation site of ErbB-1 that allows c-Cbl recruitment and down regulation (tyrosine 1045) has been previously mapped by mutagenesis to a lysosomal targeting motif [102]. In support with endosomal sorting, Cbl proteins defective in ubiquitination enhance recycling of ErbB-1 molecules, probably by inhibiting c-Cbl's action [103].

5.2. PI3K

Phosphoinositide 3-kinases phosphorylate inositol lipids at the 3′ position of the inositol ring to generate the 3-phosphoinositides PI(3)P, PI(3,4)P2 and PI(3,4,5)P3. Attempts to clarify the nature of PI3K involvement in membrane traffic in mammalian cells have been largely based on the use of inhibitors of the catalytic activity of PI3K, such as wortmannin. A post-endocytic function has been attributed to PI3K in the case of the receptor for PDGF. Inhibition of PI3K by wortmannin or mutagenesis of the PI3K docking site of the PDGF receptor resulted in altered endocytosis [104]. In both cases internalized receptors remained confined to peripheral endosomal vesicles and escaped translocation to perinuclear endosomes. How exactly PI3K drives vesicular traffic is still unknown, but a recent study revealed necessity of the kinase for structural integrity of the MVB ([105]. Unlike ErbB-3 and ErbB-4, which directly interact with PI3K, ErbB-1 seems to recruit this enzyme only indirectly, either via c-Cbl [106,107], or through ErbB-3 in a heterodimeric receptor complex [108].

5.3. Hrs

Recent research has shown that one way by which lipid kinases affect vesicular transport is by interacting with 3-phosphoinositide binding modules in a broad variety of proteins. Specifically, certain FYVE domains bind PI(3)P, whereas certain pleckstrin homology (PH) domains bind PI(3,4)P2 and PI(3,4,5)P3. One mammalian FYVE-finger protein implicated in trafficking is Hrs, a hepatocyte growth factor-regulated tyrosine kinase substrate [109], which was found to be tyrosine-phosphorylated also in an EGF-dependent manner. The localization of Hrs to an endosomal compartment seems to depend on FYVE-PI3P interactions that may cooperate with a second domain of Hrs [110,111]. Hrs is likely to be a mammalian homolog of the yeast sorter Vps27p, which is essential for vacuolar and endocytic trafficking through a pre-vacuolar compartment [112]. Accordingly, mouse cells that lack Hrs contain abnormally large early endosomes [113], and Hrs over-expression leads to the appearance of large structures containing endosomal markers [110]. These lines of evidence indicate that Hrs specifically influences the dynamics of multi-

ple endocytic compartments, which merge when the protein is overexpressed, perhaps due to promotion of vesicle aggregation or of vesicle fusion [110].

5.4. SNX-1

A yeast two-hybrid system using the core tyrosine kinase domain of ErbB-1 has identified SNX1 and implicated the protein in sorting of the receptor to lysosomal degradation [114]. SNX-1 specifically interacts with a previously identified lysosomal targeting motif, distinct from the c-Cbl's interaction site [115]. The putative sorting molecule contains a region of homology to a yeast vacuolar sorting protein, and overexpression of SNX-1 decreases the amount of ErbB-1 on the cell surface as a result of enhanced rates of constitutive and ligand-induced degradation. Recent studies revealed the existence of a large family of SNX-like molecules that are conserved in yeast and nematodes, and partly associate with the plasma membrane [116].

5.5. PLCγ

To date there is only indirect evidence for the involvement of PLCγ in ErbB-1 trafficking. Immortalized fibroblasts genetically deficient in PLCγ do not show significant effects on ErbB-1 endocytosis [117]. However, a single tyrosine, which serves as a PLCγ docking site on the receptor for the fibroblast growth factor, was found to be important for cellular trafficking [118]. Another clue for a role in endocytosis came from the observation that the SH3 domain of PLCγ is able to bind dynamin in a growth factor inducible manner [119,120].

5.6. PKC

Trans-modulation of ErbB-1 by an active PKC has been attributed to phosphorylation of a single threonine residue at the juxtamembrane domain of the receptor [121]. The modified receptor displays altered kinase activity and ligand binding affinity, and its ligand-induced down regulation is compromised [122]. PKC also affects the unoccupied receptor through enhanced internalization, which is followed by recycling back to the cell surface [123,124]. By using c-Cbl-induced ubiquitination as a marker for transfer from early to late endosomes, it has been recently shown that PKC can inhibit this process, as well as receptor down regulation and degradation [125]. Apparently, PKC-induced phosphorylation at threonine 654 is sufficient to direct incoming receptors to the recycling endosome, whereas phosphorylation at tyrosine residues, through the recruitment of c-Cbl, directs them to the MVB/late endosome. Currently it is not known how PKC activity accelerates internalization and inhibits sorting to lysosomal degradation.

6. Relationships between signalling and receptor trafficking

Ligand-induced receptor internalization has long been considered an attenuation mechanism for signal transduction. However, mounting evidence suggests more complex relationships as receptors internalized in endosomes, or immobilized at submembranal domains are capable of signalling in a surprisingly selective manner.

6.1. Signalling from caveolae

In addition to serving as a gate for entry into the cell, caveolae are the sites where multiple signalling pathways converge. Immunocytochemical, co-immunoprecipitation and cell-fractionation techniques have shown that a number of signalling proteins, including RTKs, such as the receptors for EGF and PDGF, as well as non-receptor kinases and G-proteins, are found associated with caveolae. This suggested that caveolae compartmentalize enzymatic reactions essential for signalling from the plasma membrane (reviewed in [38,126]). The hypothesis that caveolae play a crucial role in signal transduction by pre-assembling inactive signalling complexes ready for rapid activation in response to extracellular signals, is based on the following findings. Caveolin-1 can interact with the catalytic domain of many resident proteins of the caveolar fraction through the caveolin scaffold domain. These include not only ErbB-1 [44] but also c-Src and the Gα subunit of heterotrimeric G-proteins. In fact, isolated caveolae contain all essential components required for MAP kinase activation [127], and in intact cells, both PDGF [42] and EGF [41] stimulate the recruitment to caveolae of multiple signal transducing molecules, as well as the migration of the respective receptor out of caveolae [45]. Depletion of cholesterol, a major building block of caveolae, causes hyper-activation of the MAP kinase [128]. By itself, caveolin-1 is a cholesterol binding protein [129], which has a key role in controlling the level of cholesterol at the plasma membrane [130]. A dominant negative mutant of caveolin-1 is unable to mediate cholesterol trafficking to the plasma membrane, and it can block the action of H-Ras, but not K-Ras [131]. Lastly, caveolin is down-regulated and caveolae are reduced in numbers in transformed fibroblasts [132]. In accordance, conditional expression of caveolin-1 can abrogate the transformed phenotype [133], and antisense depletion of caveolin-1 in intact cells results in cell-transformation [134].

6.2. Signalling from endosomes

Evidence for the existence of highly tyrosine-phosphorylated ErbB-1 molecules in endosomes came from fractionation of rat liver [135]. This state has also been visualized directly by immunoelectron microscopy of A431 cells overexpressing the receptor [136]. The cytosolic orientation of the tyrosine-phosphorylated tail and the presence of an active receptor in endosomes for a prolonged period of time suggests that the receptor may continue to signal after internalization. Indeed, when EGF signalling was analyzed in cells whose endocytosis was inhibited by a mutant dynamin, enhanced cell proliferation was observed and analysis of signal transduction components revealed hyper-phosphorylation of both PLCγ and SHC. Unexpectedly however, MAP kinase activity was significantly reduced, along with phosphorylation of ErbB-1 [63]. Although these observations are in line with some other results [137,138], some recent observations attributed the effect on MAP kinase to inhibition of MEK endocytosis [139,140]. Another possible explanation for the attenuated signalling observed in mutant dynamin expressing cells is the unexpected loss of high affinity EGF binding sites [141].

While en route to the late endosome, ErbB-1 molecules lose the ability to stimulate the PLCγ pathway, probably because components of the pathway become inaccessible [142]. Hrs is an example for a substrate of ErbB-1 whose accessibility is enhanced, rather than diminished, upon endocytosis of the receptor [110]. p21-CIP, an inhibitor of cyclin-dependent pro-

tein kinases displays a variation on the theme; its activation by EGF occurs in an endosomal compartment, but no activation occurs following exposure of cells to another ligand of ErbB-1, namely TGFα [143]. The enhanced mitogenicity of this ligand has been attributed to dissociation of ligand–receptor complexes in the endosomal compartment, which is followed by receptor recycling [144]. Interestingly, co-expression of ErbB-2 potentiates EGF signalling to the level achieved by TGFα, due to heterodimer disintegration in the early endosome, and receptor recycling to the cell surface [145,146]. Consistent with this model, formation of ErbB-1•ErbB-2 heterodimers prevents Cbl association with ErbB-1 [147].

7. Cancer therapy: harnessing the endocytic machinery?

Overexpression of ErbB-1 is frequently detected in cancers of lung, head and neck and in brain tumors (reviewed in [148,149]). In addition, a constitutively active deletion mutant of ErbB-1 is abundant in brain tumors [150] and in other types of cancers [151]. Likewise, amplification of the erbB-2 gene is associated with a relatively aggressive subtype of breast, ovary and lung cancers [152]. These clinical observations and their relationships to poor prognosis has identified ErbB proteins as attractive targets for cancer therapy, and one such agent, a humanized monoclonal antibody to ErbB-2 is already used to treat metastasizing breast cancers [153]. Importantly, in vitro studies clearly indicate that the oncogenic action of ErbB-2 depends on its localization at the plasma membrane [154,155]. Thus, removal of ErbB molecules from the cell surface by directing them to the endocytic pathway is expected to inhibit their oncogenic potential. This may be achieved by using antibodies, modified ligands, as well as agents that interfere with translocation and stabilization of the receptors at the plasma membrane.

7.1. Immunotherapy

Extensive work in animal models has indicated that certain antibodies can effectively block the tumorigenic growth of cancer cells overexpressing ErbB-1 or ErbB-2 (reviewed in [156]). The mechanism underlying the anti-tumorigenic effect of antibodies to ErbB-2 has been attributed to the recruitment of immune cells to the tumor through the Fc portion of the antibody [157]. It is important, however, that antibodies devoid of the Fc portion are active in tumor inhibition, as long as their bivalence is maintained. Several observations are consistent with the possibility that the inhibitory effect on cancer cells is due to antibody-induced removal of the oncoprotein from the cell surface: antibodies that better down regulate ErbB-2 are superior as cancer inhibitors [158]. Likewise, examination of certain combinations of anti-ErbB-2 antibodies correlated their tumor-inhibitory effect with the ability to degrade the oncogenic receptor [159]. Consistent with this scenario, antibodies to ErbB-2 and anti-TFR antibodies co-internalize through clathrin-coated pits, coated vesicles, endosomes, and MVBs [160]. The involvement of c-Cbl in antibody-induced down regulation of ErbB-2 has been addressed by mutagenesis of the putative site of Cbl binding to this receptor [161]. The mutant receptor displayed retarded antibody-induced down regulation, suggesting that tumor-inhibitory antibodies utilize, at least in part, the c-Cbl pathway to degrade ErbB-2.

7.2. Drug-induced degradation of ErbB-2

The benzoquinoid ansamycin antibiotics geldanamycin and herbimycin A were first isolated from the culture broth of several actinomycete species [162,163], and described as inhibitors of tyrosine kinase-dependent cell growth [164,165]. These compounds display inhibitory activity toward numerous cell-lines, including those over-expressing ErbB-2. This activity is attributed to the ability of geldanamycin to induce degradation of ErbB-2 and ErbB-1, as well as of other signal transducing elements [166,167]. The mechanism underlying geldanamycin-induced protein degradation, was shown to involve the dissociation of a geldanamycin binding protein, the molecular chaperone Hsp90, or in the case of ErbB-2, its family member GRP94 [168]. A complex series of proteolytic events is involved in geldanamycin-induced ErbB-2 degradation, as was evidenced by the sensitivity of inhibitors of proteasomal and lysosomal proteases [169]. By using antibodies to both extracellular and carboxyl-terminal epitopes of ErbB-2, it was shown that geldanamycin induces fragmentation of ErbB-2 within the carboxyl-terminal region of the cytoplasmic domain, and that the resulting transmembrane fragment is degraded by a mechanism that involves the formation of intracellular vesicles of membranal origin [170].

7.3. Immunotoxins

Antibodies directed against ErbB-2 may serve as useful vehicles for targeting therapeutic agents to tumors. This approach is attractive because antibodies usually internalize together with the receptor and introduce the toxic agent into the cell [158,171,172]. Conjugates of antibodies and toxins have been used in a preclinical trial as anti-tumor agents [173]. For example, a *Pseudomonas* exotoxin lacking its cell binding domain was constructed for tumor targeting [174]. Ligands directed against ErbB proteins have also been examined as beneficial carriers. For example a betacellulin–*Pseudomonas* toxin fusion is effective against cells expressing ErbB-1, but not cells expressing ErbB-4, probably due to the limited internalizing capacity of this receptor [175]. Other studies employed antibody-containing drug-loaded liposomes that efficiently bind cancer cells and deliver cytotoxic doses of doxorubicin in a targeted manner [176], probably through an ability to internalize [177].

8. Perspectives and concluding remarks

Progression into the cell cycle requires continuous ligand occupation of growth factor receptors at the cell surface for as long as 8 h. Removal of the growth factor at any step within this critical phase, abrogates subsequent commitment to S phase entry. Recent studies that are reviewed in this volume begin to reveal the molecular basis for this requirement. These include prolonged activation of Ras, up regulation of Myc, and induction of Cyclin D. However, it is already clear that endocytosis of ligand-occupied receptors, like ErbB proteins, plays a pivotal role in controlling the duration of cell activation. The ErbB family of RTKs presents a variety of mechanisms to control kinetics of signal transduction. For example, ErbB-3 evolved as a kinase-defective receptor whose signalling, as well as sorting to degradation, requires a co-receptor. On the other hand, a major function of the most oncogenic member of the family, namely ErbB-2, seems to be prolongation of signal transduction by decelerating ligand dis-

sociation, inhibiting internalization of ErbB-2-containing heterodimeric receptors, and enhancing the rate of receptor recycling. Another layer of diversity is found at the level of the ligands. Apparently, the multiple EGF- and neuregulin-like ligands differ not only in their binding specificity and affinity but also in their kinetics of signalling. The underlying mechanisms involve differential capacity to recruit the ErbB-2 co-receptor to heterodimers, as well as disparate behavior of the many ligands while they pass through sorting barriers, such as the clathrin-coated pit and the MVB.

The sorting mechanisms are expected to be resolved in the near future. It is reasonable to assume that the major players are already known. However, their sequential engagement and mutual interactions are currently unclear. It is also conceivable that many of the signalling proteins involved in signal initiation and propagation will disclose functions as players in vesicular sorting of active RTKs. Examples include cytoplasmic tyrosine kinases (e.g., Src), adapters (e.g., Grb2 and Nck), and small GTP binding proteins (e.g., Ral). Close relationships between signal initiation and the onset of signal termination are already exemplified by the sorting molecules Eps15 and c-Cbl, two early substrates of tyrosine phosphorylation. Future research will also provide an answer to the question of signalling from the endosomal compartment, an issue that remains controversial. The exact role of second messengers like calcium ions, diacylglycerol and inositol phospholipids will probably become clearer. Likewise, the extent of similarity between yeast and animal cells will have to be defined. One relevant example is the role, if any, of receptor ubiquitination as an internalization signal. Partitioning of receptors among the various endocytic pathways is another important question. Presumably, constitutive and ligand-activated pathways overlap to some extent, but molecular machineries that determine their remarkably different rates are still unknown. The relationships between these two pathways and the stress-induced endocytic response, which also involves extensive receptor endocytosis and degradation, will probably emerge.

Comprehensive understanding of the mechanisms that negatively regulate signal transduction by RTKs have wide range of implications; from embryonic development to pathological states, like wound healing, hyper-proliferative diseases and cancer. Potentially, genes involved in receptor down regulation may act as tumor suppressors. Moreover, drugs that enhance down regulation or interfere with sorting decisions are clinically useful. Indeed, humanized antibodies to ErbB-2, similar chimeric monoclonal antibodies to ErbB-1, as well as tyrosine kinase inhibitors and drugs that inhibit heat shock proteins, are already in different phases of clinical testing or application. More molecular targets for therapeutic intervention will likely emerge from future studies of receptor down regulation.

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The deaf and the dumb: the biology of ErbB-2 and ErbB-3

Ami Citri, Kochupurakkal Bose Skaria, and Yosef Yarden*

Department of Biological Regulation, The Weizmann Institute of Science, Rehovot 76100, Israel

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Abstract

ErbB-2 (also called HER2/neu) and ErbB-3 are closely related to the epidermal growth factor receptor (EGFR/ErbB-1), but unlike EGFR, ErbB-2 is a ligandless receptor, whereas ErbB-3 lacks tyrosine kinase activity. Hence, both ErbB-2 and ErbB-3 are active only in the context of ErbB heterodimers, and ErbB-2 · ErbB-3 heterodimers, which are driven by neuregulin ligands, are the most prevalent and potent complexes. These stringently controlled heterodimers are repeatedly employed throughout embryonic development and dictate the establishment of several cell lineages through mesenchyme-epithelial inductive processes and the interactions of neurons with muscle, glia, and Schwann cells. Likewise, the potent combination of signaling pathways engaged by the heterodimers drives an aggressive phenotype of tumors of secretory epithelia, including breast and lung cancers. This review highlights recent structural insights into the mechanism of ligand-induced heterodimer formation, and concentrates on signaling pathways employed by ErbB-2 and ErbB-3 in normal and in malignant cells.

Introduction

The ErbB-2 · ErbB-3 heterodimer constitutes the pinnacle of ErbB receptor evolution, demonstrating the capacity of evolution to form an extremely potent signaling module from a pair of singly inactive individual proteins. The diversification of the ErbB family during evolution, from one receptor/ligand in worms, through one receptor/multiple ligands in flies, to four receptors and multiple ligands in mammals, has created a network capable of precise signaling in a widely divergent fashion [1]. Thus, through utilization of defined receptor pairs, activated by specific ligands, a graded signaling potency can be obtained, leading to a precise cellular outcome. An additional level of signaling diversity is obtained through differential activation of distinct signaling molecules downstream of each receptor. The capacity to form precise signaling is best exemplified by the ErbB-2 · ErbB-3 dimer. ErbB-3 is an impaired kinase due to substitutions in critical residues in its catalytic domain [2], and thus can signal only in the context of a receptor heterodimer. In addition, it is now clear that ErbB-2 is devoid of an activating ligand [3] and can act only in the context of a heterodimer with a ligand-bound receptor. In stark contrast to their apparent disabilities, this receptor pair forms the most potent signaling module of the ErbB-receptor family in terms of cell growth and transformation [4,5]. That the most potent signaling module is formed by partners that are incapable of productively signaling in isolation suggests that evolutionary forces formed these mechanisms as a measure to tightly control the output of the network. As will be described below, the basis for the potency of signaling by the ligand-activated ErbB-2 · ErbB-3 heterodimer lies in the fact that this dimer has the capacity to signal very potently, both through the Ras-Erk pathway for proliferation, and through the phosphatidylinositol-3′-kinase (PI3K)-Akt pathway for survival. In addition, this receptor dimer evades downregulation mechanisms, leading to prolonged signaling [6,7].

ErbB-2 and ErbB-3 as determinants of cell lineages

Organ morphogenesis is controlled, at least in part, by multiple polypeptide factors that transmit signals between neighboring cells. The ErbB-receptor family plays a pivotal role in cell lineage determination in a variety of tissues, including mesenchyme-epithelial inductive processes in epithelial organs (reviewed by Burden and Yarden [8]).

* Corresponding author. Fax: +972-8-9342488.
E-mail address: yosef.yarden@weizmann.ac.il (Y. Yarden).

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ErbB-2 and ErbB-3, as well as ErbB-1, are expressed in most epithelial cell layers, and mesenchymal cells are a rich source of ErbB-ligands, both neuregulins (NRGs) and epidermal growth factor (EGF)-like ligands. During development of the human fetus, ErbB-2 is found in the nervous system, developing bone, muscle, skin, heart, lungs, and intestinal epithelium [9]. Likewise, ErbB-3 is found in cells of the gastrointestinal, reproductive, and urinary tracts, as well as the skin, endocrine, and nervous systems [10].

Cardiac development

The roles of ErbB-2 and ErbB-3 in development are best delineated by the phenotypes of mice in which the *erbB-2* and *erbB-3* genes have been inactivated, as well as the phenotype of *nrg-1*$^{-/-}$ mice. Essentially, the cardiac phenotypes of NRG-1 null mice and ErbB-2 null mice appear similar, and represent the sum of the changes observed in ErbB-3 and ErbB-4 null mice [11]. The most striking phenotype of ErbB-2 null mice is early death at mid-gestation (E10.5) due to malformation of trabeculae in the heart [12], a phenotype shared with ErbB-4 and NRG-1-defective mice [13,14]. NRG-1 is expressed in the endocardium, an endothelial ventricular lining, while ErbB-2 and ErbB-4 are expressed in the myocardium, which is the underlying muscular portion of the ventricle and atrium. Mice expressing a kinase-defective mutant of ErbB-2 instead of wild-type receptor die at mid-gestation and express the same spectrum of embryonic defects seen in ErbB-2-defective mice, demonstrating that the catalytic activity of ErbB-2 is essential for its function in embryogenesis [15]. While the process of heart trabeculation does not appear to require ErbB-3, this receptor is essential for normal cardiac development [16]. Expression of ErbB-3 is restricted to the mesenchyme of the endocardial cushion, which develops into the heart valves. In *erbB-3*$^{-/-}$ mouse embryos the trabeculation occurs in a delayed but otherwise normal fashion, but their atrioventricular valves are rudimentary and thinned, leading to death at E13.5. NRG-1 plays a crucial role in this inductive process [17], but ErbB-2 does not appear to be involved, since it is not expressed in the embryonic endocardial cushion. Targeted inactivation of ErbB-2 in ventricular cardiomyocytes led to a severe dilated cardiomyopathy, causing cardiac dysfunction by the second postnatal month, suggesting that ErbB-2 signaling is essential for adult heart function [18,19]. The cardiac phenotypes expressed by mice carrying mutations in *erbB-2* and *erbB-3* demonstrate the basic principle underlying the function of the encoded receptors, i.e., heterodimerization is essential for induction of receptor's function.

Glial and neuronal cell development

In addition to their roles in synapse formation and neuronal development, NRGs appear to act as major regulators in the development of myelinating cells in the peripheral (Schwann cells) and central (oligodendrocytes) nervous systems [20]. Mice individually mutant for *erbB-2, erbB-3,* or *nrg-1* display a failure in neural crest development, leading to impaired formation of the sympathetic nervous system [21]. The action of NRG-1, secreted by neurons, is essential for both the proliferation and the maturation of Schwann cells and oligodendrocytes. Accordingly, ErbB-3 is expressed in the responding myelinating cells, and wherever investigated, the effect of NRG-1 appears to be mediated by ErbB-3, acting in the context of ErbB-1 or ErbB-2. While NRG-1 is important throughout oligodendrocyte differentiation, using complex transgenic systems it has been established that ErbB-2 is important for late oligodendrocyte differentiation, and for the development of myelin [22,23]. In addition, it has also been demonstrated that a heterodimer of ErbB-2 and ErbB-3 is the active receptor in Schwann cell differentiation [24,25]. Consistent with observations made in vitro with cultured cells, in *erbB-3*-defective mice Schwann cells fail to develop, and most sensory and motor neurons subsequently die (reviewed by Davies [26]).

Mammary gland development

The mammary gland is one of the few organs in which major morphogenetic changes take place after birth. Two phases of morphogenesis occur, i.e., during puberty, and during pregnancy. In contrast to their ligands, which are expressed in defined time windows, all ErbB receptors are expressed throughout most developmental stages of the mammary gland (reviewed by Troyer and Lee [27]). In organ cultures of mammary glands, NRG-1 stimulated lobuloalveolar budding and the production of milk proteins [28]. In addition, branching morphogenesis and lobulo-alveolar differentiation of the mammary gland could be abolished by blocking expression of endogenous NRG. However, in transgenic animals with targeted expression of NRG-1 in the mammary gland, persistence of terminal end buds was observed, suggesting that NRG-1 inhibits signals that normally lead to the terminal differentiation of these structures [29]. Consistent with a role for ErbB-2 as a coreceptor, transgenic mice expressing a dominant-negative ErbB-2 in the mammary gland display normal ductal growth, but have defective lobuloalveoli and reduced milk protein secretion [30]. Due to coexpression of all ErbBs and many of their ligands, more experimental models will be needed to resolve the exact role of ErbB-2 · ErbB-3 heterodimers in mammary development.

Formation of ErbB-2 · ErbB-3 heterodimers: structural insights

While it is clear that dimerization of ErbB proteins is crucial for signaling (reviewed by Heldin and Ostman [31]), the underlying mechanism remained elusive until very recently. Three published structures of the extracellular domains of ErbB family receptors [32–34] have recently pro-

vided a framework for understanding the large amount of data that have accumulated over the years with respect to ligand binding and receptor activation. ErbB receptors share a high degree of primary sequence homology. Four subdomains have been identified in the extracellular domain; subdomains I (L1) and subdomain III (L2) mediate ligand binding [35,36], while according to the recently published structures, the cysteine-rich subdomains II (S1) and IV (S2) play a role in receptor dimerization. Most previous lines of evidence relate to the interaction of EGF with ErbB-1 (reviewed by Groenen et al. [37]). For example, several lines of evidence concluded that EGF binds ErbB-1 with a 1:1 stoichiometry, perhaps through a cleft formed by subdomains L1, L2, and S1 [38]. In addition, EGF binding to an isolated extracellular domain has been associated with a conformational change [39], but the exact mechanism of dimerization remained unknown until very recently.

Earlier lines of evidence

Mutagenesis of both NRG-1 [40] and EGF [36] concluded that these ligands bind their receptors in a bivalent manner. On the other hand, biophysical analyses of soluble ErbB-1 suggested dominance of a 2:2 ligand:receptor configuration [41]. Last, fluorescence imaging microscopy suggested that ErbB-1 exists in a predimerized state, but ligand binding induces a rotational rearrangement of the monomeric subunits [42]. This view is consistent with single molecule imaging of ErbB-1, which concluded that EGF first binds to predimerized receptors, and then a second EGF molecule binds to the 1:2 complex [43].

Insights from crystal structures

The crystal structures of ligand-bound ErbB-1 confirmed both bivalent ligand binding and a final 2:2 complex [33,34], consistent with the implications of the structure of a nonliganded ErbB-3 [32]. In the crystal structures of ErbB-1 and ErbB-3, L1 and L2 have a β-helical fold, while the S1 and S2 subdomains have an extended structure held together by disulfide bonds. The S1 subdomain traverses along one face of the β-helix of the L1 domain with a large interface conferring rigidity to the juxtaposition of the L1-S1 domains. Two structures of ErbB-1 have been described, one bound to EGF [33] and the other bound to transforming growth factor-α (cTGFα) [34]. In both cases, bivalent ligand binding to the L1 and L2 domains is observed and the ligand holds these domains in a rigid conformation. A long β-hairpin, termed the "dimerization loop" extends out of S1 and was found to be the primary mediator of dimerization of two monomeric ErbB-1 molecules. In the dimer, the two ligand-bound monomers approach each other back-to-back and the dimerization loop of one receptor extends deep into the dimer partner. The tip of the loop contacts residues in the L1 and L2 domains, contributing to dimer stabilization.

The role of the S2 domain

The published structures of ErbB-1 did not resolve the structure of S2. However, short cyclic peptides identical in sequence to the C-terminus of the S2 domain of ErbB-2 were found to bind the receptor as well as inhibit receptor signaling [44], suggesting that the S2 domain may be involved in dimerization. In addition, S2 has been previously demonstrated to reduce ligand binding affinity, suggestive of an intramolecular inhibitory interaction [45]. In the structure of the unliganded ErbB-3, an intramolecular interaction between the dimerization loop (from S1) and a β-hairpin protruding out from the S2 domains holds the receptor L domains far apart in an open conformation [32]. Taken together with the conformation of the ligand-bound ErbB-1, these lines of evidence raise the possibility that unengaged ErbB-1 and ErbB-4 may exist in a similar locked conformation on the cell surface. According to this model, ligand binding to domains L1 and L2 induces rotation of the rigid L1-S1 domains with respect to the L2 domain around the S1-L2 linker, and tightly bridges the L1 and L2 domains. Consequently, this conformational change exposes the dimerization loop of the receptor, rendering monomeric, ligand-bound receptors amenable to dimerization.

Dimers containing ErbB-2

Although ErbB-2 binds no known ligand, when recruited into heterodimers it increases ligand binding affinity [46,47]. It is also the favored receptor for heterodimerization [48,49]. Interestingly, in the structure of ErbB-2, unlike the other receptors in the family, a strong interaction between L1 and L2 domains was observed, mimicking the ligand-bound form in the ErbB-1 structure (A.W. Burgess, personal communication). This interaction involves regions corresponding to ligand-binding sites in the L1 and L2 domains of ErbB-1, rendering ErbB-2 incapable of binding ligands. The consequence of the L1-L2 interaction in ErbB-2 is a constitutively extended conformation of the dimerization loop. Hence, the promiscuous behavior of ErbB-2 and its inability to bind EGF-like ligands seem inherent to its structure. Fig. 1A presents a model for the formation of ErbB-2 · ErbB-3 heterodimers. This model was conceived based on the known structures of ErbB-3 and ErbB-1, in combination with previously described lines of evidence. Accordingly, the ligandless ErbB-2 is predisposed for dimerization because its dimerization loop is preextended. On the other hand, ligand binding to ErbB-3 releases a locked conformation and extends the dimerization loop. Finally, within the dimer, both S1 and S2 domains of each receptor form two distinct interfaces, which stabilize the heterodimer. An alternative view would suggest that a preformed heterodimer assumes a twisted active conformation upon binding of a ligand [42].

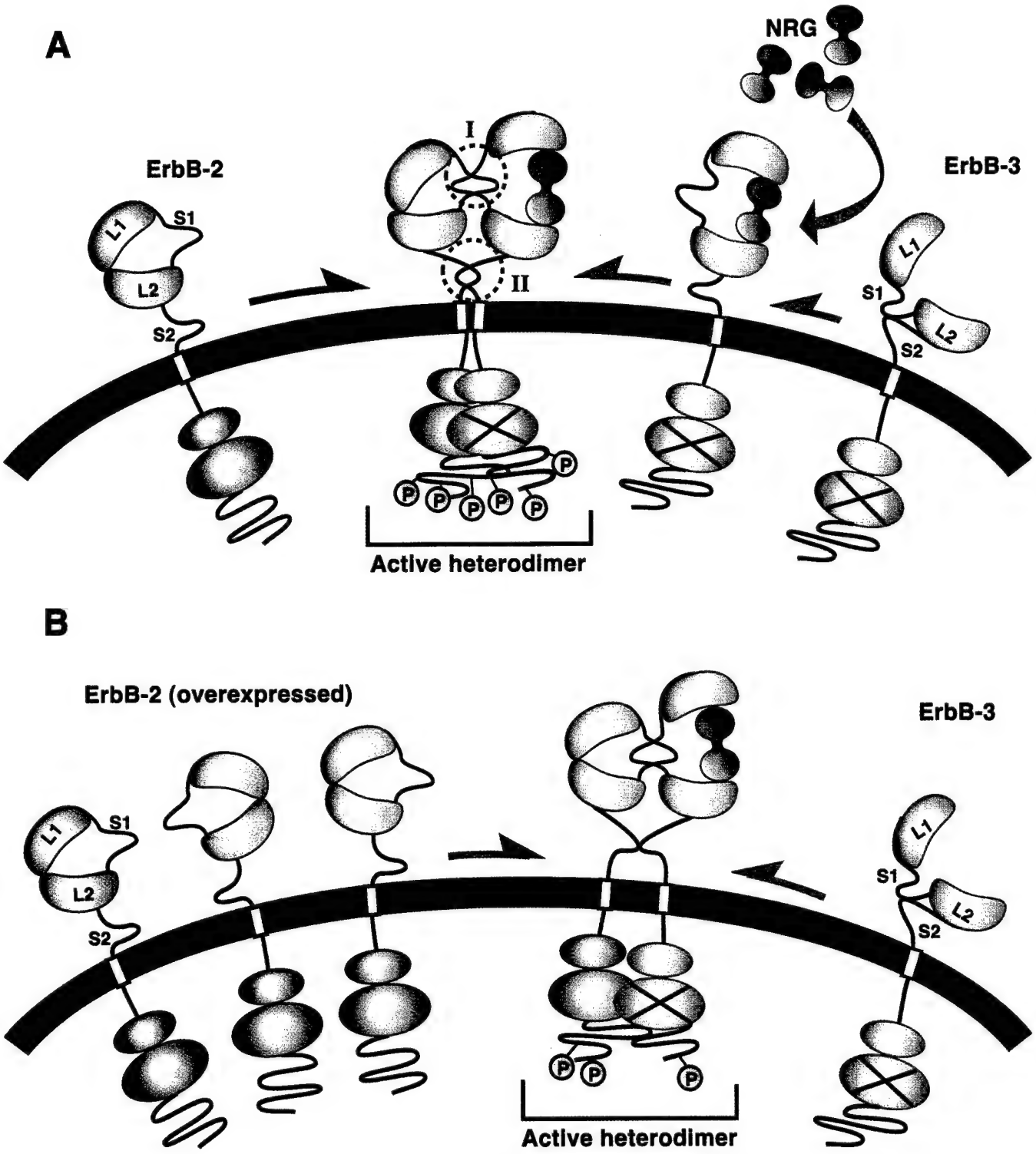

Fig. 1. Schematic representation of ligand-induced receptor heterodimers. The proposed mechanism of neuregulin-induced ErbB-2 · ErbB-3 heterodimers is depicted. (A) The extracellular domains are represented by two cysteine-rich domains (S1 and S2) and two cysteine-free, ligand-binding domains (L1 and L2). ErbB-3 (blue) exists on the cell surface in an autoinhibited conformation resulting from the interactions between the S1 and S2 domains. Bivalent binding of neuregulin (NRG, represented here as a red dumbbell) to the L1 and L2 domains of ErbB-3 rearranges the conformation of the extracellular domain, leading to protrusion of the S1 dimerization loop. In the case of ErbB-2, the intramolecular interaction between L1 and L2 results in a preextended conformation of the S1 dimerization loop. Dimerization between a ligand-bound ErbB-3 and an ErbB-2 molecule is mediated primarily by the dimerization loop (dotted circle I), with additional possible contributions from the loop in the S2 domain (dotted circle II). Additional stabilizing interactions between the transmembrane and kinase domains may also play a role. While the simplest scenario is depicted, receptor trimers and tetramers have also been proposed. (B) Overexpression of ErbB-2 at the cell surface may spontaneously recruit an autoinhibited ErbB-3 into heterodimers. The formed dimers may assume the ligand-induced conformation, resulting in weak but prolonged receptor activation. Alternatively, spontaneous homodimers formed upon overexpression of ErbB-2 cannot be excluded.

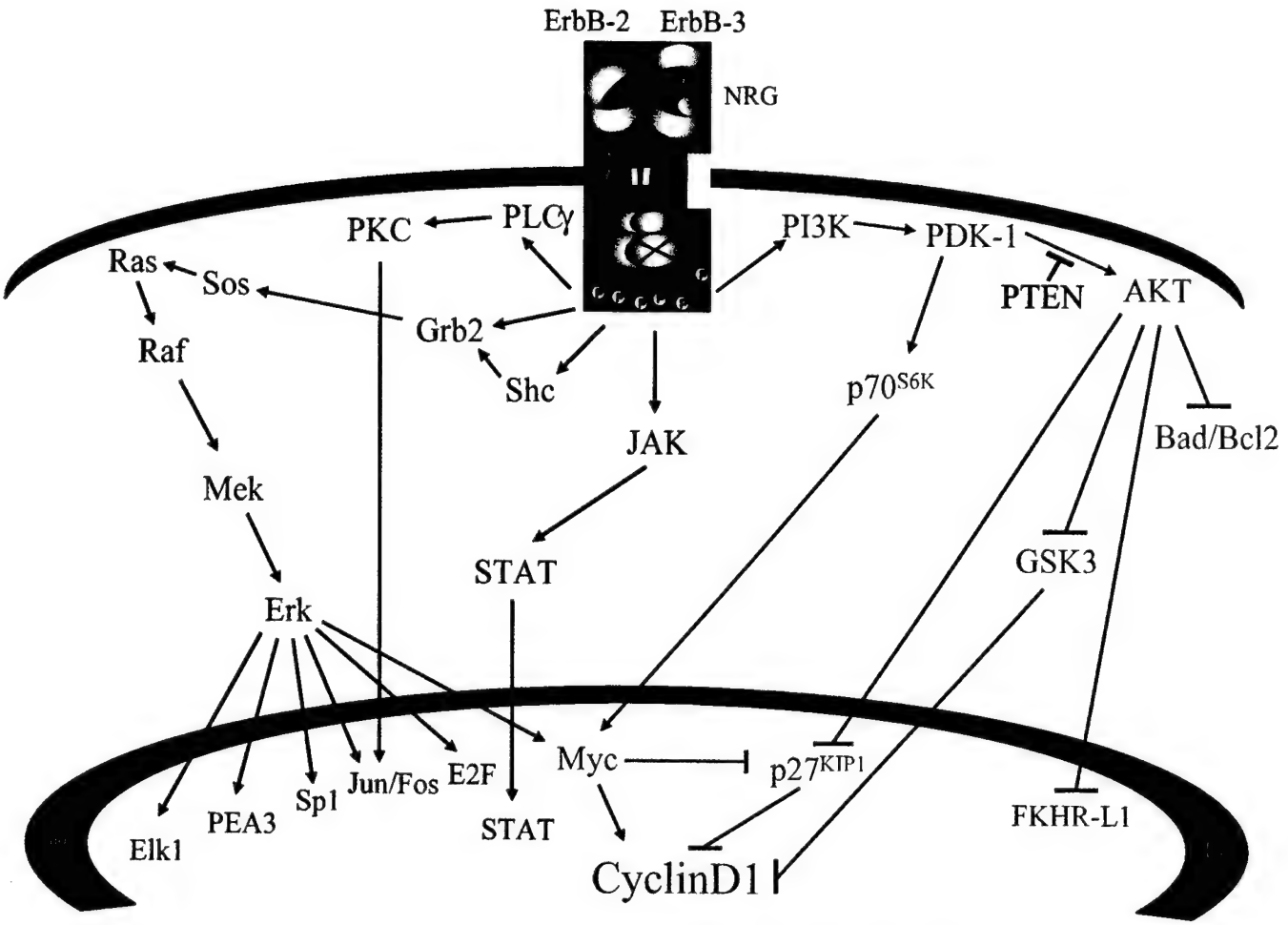

Fig. 2. The major signaling pathways stimulated by ligand-activated ErbB-2 · ErbB-3 heterodimers. Ligand-induced formation of the ErbB-2 · ErbB-3 heterodimer (see Fig. 1) at the cell surface leads to activation of several major pathways of signal transduction. This process results in enhanced cell survival and mitogenicity, and its deregulation can lead to tumorigenesis. Erk activation by the Ras-Raf pathway leads to cell proliferation through the activation of a number of nuclear targets, including Elk1, PEA3, Sp1, AP1, and the c-Myc oncoprotein, which is a major transcription factor and regulator of cell cycle progression. Another pathway is the PI3K-Akt pathway, activation of which results in enhanced antiapoptotic and prosurvival signals, through inhibition of the proapoptotic proteins Bad, GSK3, and the transcription factor FKHR-L1. In addition, the PLCγ and the JAK-STAT pathways are indicated, with their resulting enhancement of transcription leading to cell proliferation. A major player acting downstream of ErbB-2 · ErbB-3 is cyclin D1. As indicated, a number of pathways lead from the receptors to enhanced activation of cyclin D1, thereby promoting cell cycle progression. Note that the outcome of activation of these different signaling pathways depends on the cellular context, and can vary from proliferation to differentiation, migration, and even induction of apoptosis.

Dimerization driven by receptor overexpression?

Amplification of the *erbB-1* and *erbB-2* genes is a common theme in epithelial cancers, and breast cancer patients whose tumors overexpress ErbB-2 better benefit from treatment with anti-ErbB-2 antibodies (reviewed by Yarden and Sliwkowski [1]). In model systems, overexpression of ErbB-1 is oncogenic but only in the presence of a ligand, whereas overexpression of ErbB-2 is transforming even in the absence of a ligand [50,51]. Likewise, although ErbB-2 bearing an activating point mutation is transforming in the absence of a ligand [52], the presence of another member of the ErbB family seems essential [53]. Hence, activation of ErbB-2 may not occur through formation of its own homodimers. Instead, ErbB-2-containing heterodimers may form when ErbB-2 is overexpressed. The model presented in Fig. 1B explains how heterodimerization of ErbB-2 with ErbB-3 may occur upon overexpression of ErbB-2, and a similar mechanism may underlie the oncogenic potential of coexpressed ErbB-1 and ErbB-2 [54].

Signal transduction by ErbB-2 · ErbB-3 dimers

Cancers do not necessarily arise as a linear result of an increased rate of cellular proliferation, but rather the balance between cell division and apoptosis is the crucial factor [55]. This principle is demonstrated by the oncogenic potential of the ErbB-2 · ErbB-3 dimer; this receptor signals through both the mitogen-activated protein kinase (MAPK) pathway, which drives cell proliferation and additional processes, and through the PI3K/Akt pathway, which primarily drives cellular survival and antiapoptotic signals (Fig. 2).

The ErbB-2 · ErbB-3 complex is the most active ErbB dimer

Comparative analyses of individual homodimers and heterodimers of ErbB proteins introduced into naive, ErbB-free cells, revealed that ErbB-3 is signaling defective, whereas ErbB-2 cannot be stimulated by any known ligand [4,56,57]. Nevertheless, ErbB-2 can enhance and prolong signaling by many EGF-like ligands [46]. In addition, within the hierarchical dimerization network of ErbB receptors, ErbB-2 represents the preferred heterodimerization partner of all other ErbB receptors, and the preferred dimerization partner of ErbB-2 is ErbB-3 [49]. The remarkable signaling potency of ErbB-2 · ErbB-3 dimers is the outcome of several features outlined below:

Slow rate of ligand dissociation

ErbB-2 enhances the affinity of the direct receptors for their ligands [47,49,58,59]. This effect seems to be shared by all EGF-like ligands and may reflect the promiscuous behavior of ErbB-2 as a heterodimerization partner [3]. While ligand affinity is determined by both the rate of association with the receptor, as well as by the rate of complex dissociation, only the latter parameter is modulated by ErbB-2 [46].

Relaxed specificity to EGF-like ligands

When ErbB-2 joins ErbB-3 it not only confers higher binding affinity, but also widens the spectrum of ligand binding toward neuregulins and EGF-like ligands [60,61]. This attribute is not a simple outcome of increased affinity, because some ligands, e.g., EGF and betacellulin, gain recognition, while others, e.g., TGFα, do not. Although the underlying mechanism remains unknown, it seems that this activity is due to an intrinsic capability of ErbB-2, because ErbB-2 · ErbB-4 heterodimers demonstrate a similar phenomenon [62].

Evasion of endocytosis

The major pathway leading to inactivation of signals emanating from ligand-activated growth factor receptors is an endocytic process that sorts active receptors to degradation in lysosomes (reviewed by Waterman and Yarden [63]). In the case of ErbB-1 this process is robustly regulated by an E3 ubiquitin ligase, called c-Cbl, that binds a specific phosphotyrosine of ErbB-1, thereby enhancing receptor ubiquitination and subsequent sorting to endocytosis and degradation [64–68]. In contrast, ligand-induced endocytosis of other ErbBs is slower [4,6], probably because these receptors are only weakly coupled to c-Cbl [48,69,70]. Consequently, ligands of ErbB-3 undergo relatively slow degradation [71], whereas ErbB-3 itself evades downregulation and undergoes recycling to the plasma membrane [72]. Defective endocytosis and enhanced recycling characterize not only the ErbB-2 · ErbB-3 dimer, but also other ErbB-2-containing heterodimers [73–75].

Coupling to potent signaling pathways

Within a ligand-occupied ErbB-2 · ErbB-3 dimer, transphosphorylation takes place and as a result, several phosphotyrosine residues located in the carboxyl-terminus of each receptor undergo phosphorylation [5]. Remarkably, a single site of ErbB-2, which recruits Shc and couples to the MAPK pathway, is sufficient for cell transformation [76], and multiple sites of ErbB-3 are able to recruit PI3K [77], thereby activating the Akt pathway. Normally ErbB-3 is restrained through its lack of kinase activity, which is compensated by heterodimerization. The strong signaling potential of this receptor is exemplified by an artificial fusion protein containing the kinase domain of ErbB-1 and the carboxyl-terminal tail of ErbB-3 [7]. This chimera is extremely mitogenic because it strongly couples to PI3K, avoids c-Cbl and endocytosis, and transmits prolonged signals through the Shc-MAPK pathway. In conclusion, several mechanisms allow ErbB-2 and ErbB-3 to escape normal constraints, and their combined dimer is characterized by ligand promiscuity and potent signaling.

Signaling pathways activated by ErbB-2 and ErbB-3

Several major pathways are stimulated upon activation of ErbB-2 and ErbB-3. These are MAPK [76], PI3K [77,78], phospholipase-Cγ (PLCγ; [79,80]), protein kinase C, and the Janus kinase (Jak-STAT; [81]). Remarkably, it appears that the heterodimers avoid coupling to Grb2 and the Ras-specific GTPase-activating protein (Ras-GAP; [82]), effectors that can also negatively regulate mitogenic signals [83,84].

MAPK pathway

Stimulation of Erk occurs upon ligand-induced activation of a receptor dimer, which binds Grb2 through a phosphorylated tyrosine-based consensus site, or indirectly, through interaction with Shc (reviewed by Marshall [85]). Grb2 is associated with Sos, a guanine nucleotide exchange factor specific for Ras, and Sos activates Ras by exchanging GDP for GTP. In the GTPase active state, Ras interacts with Raf and stimulates a linear kinase cascade culminating in activation of Erk/MAPK. Erk phosphorylates a variety of cytoplasmic and membranal substrates, and is rapidly translocated to the nucleus, where it activates a number of transcription factors including Sp1, PEA3, E2F, Elk1, and the AP1 transcription factor formed by Jun and Fos.

PI3K/AKT

Activation of PI3K occurs through binding of the regulatory p85 subunit of the lipid kinase to a phosphotyrosine consensus site on the receptor, leading to allosteric activation of the p110 catalytic subunit. p110 activation produces phosphatidylinositol-3,4,5-trisphosphate [PtdIns(3,4,5)P_3] from PtdIns(4,5)P_2 within seconds, and delayed production of PtdIns(3,4)P_2 through the action of 5′-inositol phosphatases. The effects of polyphosphinositides in the cell are

mediated through the action of two lipid-binding domains, the FYVE domain, which binds to PtdIns(3)P, and the PH domain, which binds to PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3. The PH domain-containing proteins PDK-1 (reviewed by Tokev and Newton [86]) and Akt/PKB are key mediators of PI3K signaling, and both are essential for the transforming effects of PI3K (reviewed by Blume-Jensen and Hunter [87]). Upon production of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 following activation of PI3K by the ErbB-2·ErbB-3 receptor dimer, Akt is recruited to the plasma membrane by its PH domain, and is phosphorylated by PDK-1. Akt phosphorylation causes its activation and translocation to the nucleus, where it acts upon its targets, which are either regulators of apoptosis or of cell growth (reviewed by Meier and Hemmings [88] and Cantley [89]). The tumor suppressor PTEN is a lipid phosphatase, which dephosphorylates the 3′-OH position of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, thereby reverting the activity of PI3K, and downregulating the activity of PDK-1 and Akt.

PLCγ

Activation of PLCγ by ErbB-2, rather than by ErbB-3 [82], occurs through its SH2-mediated recruitment to phosphorylation-dependent docking sites on ErbB-2, as well as recruitment through its PH domain to the plasma membrane. In its phosphorylated active form, PLCγ hydrolyzes PIP_2 ($PI4,5P_2$; phosphatidylinositol 4,5 biphosphate) into IP_3 (inositol 1,4,5-triphosphate), and diacylglycerol. IP_3 activates the release of calcium from intracellular stores, and thereby activates calcium/calmodulin-dependent kinases, as well as additional pathways, and it collaborates with diacylglycerol to stimulate protein kinase C (reviewed by Karin and Hunter [90] and Hunter [91]).

Cell adhesion molecules

CD44, a surface glycoprotein implicated in cell adhesion and motility, has been found to complex with ErbB-2 in ovarian and Schwann cells. CD44 coimmunoprecipitates with ErbB-2 and ErbB-3, and may potentiate the response to NRG-1 by facilitating receptor heterodimerization [92,93]. An additional positive modulator is the MUC4 sialomucin, which forms a complex with ErbB-2 in a number of tissues. MUC4 has been suggested to act as a modulator of the signaling activity of ErbB-2, inducing specific phosphorylation of ErbB-2, and potentiating NRG signaling [94].

Cyclin-dependent kinases and cell cycle regulation

Hyperactivated signaling through ErbB receptors results in deregulation of the cell cycle homeostatic machinery and upregulation of complexes containing cyclin D and cyclin-dependent kinases (CDKs), resulting in enhanced proliferation and malignant transformation. Cyclin D1 is a central effector of signaling by ErbB-2 and ErbB-3, and has been implicated as a major player in breast cancer acting to promote cell cycle progression, through activation of its catalytic partners CDK4 and CDK6 (reviewed by Harari and Yarden [95]). Both MAPK and PI3K can modulate the activity of cyclin D1 downstream of ErbB-2 [96]. The MAPK pathway has been implicated in transcriptional upregulation of cyclin D1 through the Sp1, AP1, and E2F transcription factors [97,98], and posttranslational stabilization of cyclin D1 can be conferred by its phosphorylation by Akt [99]. Additional key regulators of CDK function are $p27^{KIP1}$ and $p21^{Waf1}$, previously implicated as CDK inhibitors, but currently suggested to produce both activating and inhibiting functions, depending on their expression levels. Thus, ErbB-2/Neu-induced, cyclin D1-dependent transformation is accelerated in p27-haplo-insufficient mammary epithelial cells, but impaired in p27 null cells [100], and ErbB-2 and ErbB-3 function together to stimulate mitogenic signaling networks by Akt- and c-Myc-dependent sequestration of $p27^{KIP1}$, leading to deregulation of the G1-S cell cycle transition [101].

Negative regulators

RALT is a feedback inhibitor of ErbB-2 signaling whose expression is induced upon stimulation of the MAPK pathway [102]. RALT binds to the kinase domain of ErbB-2, and inhibits Erk activation and cellular transformation driven by ErbB-2 [103]. A different mode of ErbB-2 inhibition is exemplified by Herstatin, an alternatively spliced form consisting of a segment of the extracellular domain of ErbB-2 fused to a novel carboxyl-terminus. Herstatin binds to ErbB-2 and inhibits heterodimerization and activation of ErbB-3 [104]. A similar type of naturally secreted protein consisting of the extracellular domain of the receptor has been described for ErbB-3. This secreted protein (p85-s) inhibits NRG-stimulated activation of ErbB-2, ErbB-3, and ErbB-4 through sequestration of the ligand [105]. An additional mode of regulation of ErbB-2 is mediated by the action of the heat shock proteins Hsp90 and Hsp70, and their associated E3-ubiquitin ligase CHIP, which acts to promote the ubiquitylation of ErbB-2, and its subsequent degradation [106,107]. This mechanism of cellular regulation bears significant potential for pharmaceutical intervention in ErbB-2-dependent tumors.

Clinical implications of the cooperation between ErbB-2 and ErbB-3

Several lines of evidence derived from animal models and in vitro systems imply that ErbB-1 can transform naive cells only when one of its ligands, primarily TGFα, is available [50,108]. Consistent with this notion, analyses of human tumors from gastric, breast, pancreatic, and other origins indicated that autocrine loops underlie poor prognosis of the relevant ErbB-1-overexpressing tumors (reviewed by Salomon et al. [109]). Another critical partner of ErbB-1 is ErbB-2, as their coexpression drives oncogenesis in model systems [54,57]. In analogy to ErbB-1, when singly expressed, ErbB-3 is nonmitogenic [4], but together with

ErbB-2 and a neuregulin it transmits not only potent mitogenic signals [4,56,57], but also signals for tumorigenic growth [5,57,110]. As discussed earlier, whether or not ErbB-2 can transform cells when singly expressed is yet unclear; while the catalytic activity of this receptor is relatively high, even in the absence of a stimulating ligand [111], a transforming mutant, whose catalytic activity is constitutively elevated [112], loses its oncogenic potential when expressed in an ErbB null cellular environment [53].

ErbB-2 is overexpressed in a large proportion of breast and ovarian tumors (20–30%), primarily due to gene amplification [113,114]. ErbB-2 appears not to be expressed in benign tumors before the onset of malignant disease [115], but overexpression is maintained in metastatic lesions. The prognostic significance of ErbB-2 overexpresion in human cancer has been extensively reviewed [116–118], and therefore we limit our discussion to the available clinical data related to coexpression of ErbB-2 and neuregulin receptors. Expression of the high affinity neuregulin receptor ErbB-4 is relatively variable in carcinomas, and it may associate with a differentiated phenotype and better prognosis of breast tumors [119]. In contrast, coexpression of ErbB-2 with ErbB-4 in childhood medulloblastoma predicts poor prognosis [120]. Significantly, when singly analyzed in brain tumors, expression of neither receptor was predictive. Coexpression of ErbB-2 with the low affinity neuregulin receptor ErbB-3 may be similarly relevant to epithelial tumors. However, detection of such an association is potentially blurred by the following two factors: First, ErbB-3 is expressed in the majority of tumors of the breast, skin, ovary, and gastrointestinal tract [121–124]. Second, the respective gene shows no amplification or rearrangements. Nevertheless, along with reports that failed detecting association between ErbB-3 and clinical outcome, several studies associated ErbB-3 expression with pathological parameters. Examples include advanced non-small cell lung carcinomas in which high ErbB-3 predicted shorter patient survival [125], early invasive ovarian lesions [126], hepatocellular carcinomas [127], oral squamous cell carcinomas [124], and bladder cancers in which coexpression correlated with patient survival [128].

In conclusion, there are clinical indications supporting the concept emerging from in vitro studies that neither ErbB-2 nor ErbB-3 can be considered as stand-alone receptors. Future studies must also address the presence of neuregulins, because unlike ligands of EGFR, which seem to control autocrine loops in human cancers [129], neuregulins may form paracrine loops in breast [130] and prostate cancer [131]. Another variable is the occurrence in tumors of secreted ErbB-3 isoforms capable of neuregulin binding [105].

Perspectives

Peaking in the recent months, the past 15 years have been highly instructive as to the basic principles underlying the action of ErbB receptors, and in describing their relevance to tumorigenesis, thereby opening windows for therapeutic opportunities. Thus, the basic principle of receptor heterodimerization has taught us that the context in which a receptor functions is crucial for predicting the resulting signal. This context is beginning to be extended to the interaction of the ErbBs with receptors of other families and to cross-talk between signaling pathways (reviewed by Carpenter [132]). The recently resolved structures of the ectodomains of ErbB receptors will be instructive not only for understanding how ligands promote receptor dimers, but also help develop peptidergic and other ErbB blockers. It is interesting that a naturally occurring antagonistic ligand exists in flies (i.e., Argos [133]), but attempts to generate a similar blocker of mammalian ErbBs have failed so far.

Apart from spaciotemporal regulation of the expression of ligands, receptors, and downstream effectors, it is currently unclear how different neuregulins elicit unique responses, although they utilize similar receptor combinations. Differences in affinity of different ligands for the same receptor combination have been suggested to be a deciding factor in signal outcomes. Another possibility is the amplification of subtle differences in the conformations of the dimers induced by different ligands, when higher order oligomers of the receptors are formed [134]. Would low affinity viral ligands induce an open conformation of the dimerization loop, or do they exploit the small fraction of predimerized receptors by rearranging them to potentiate signaling [135]? If a ligand binds to only one receptor, how is the identity of the receptor dimer decided? What is the mechanism by which heterodimerization is preferred over homodimerization? And, how is the dimerization signal transferred through the membrane, leading to activation of the kinase domain? These and other questions will most likely await the resolution of the structure of a receptor heterodimer in the context of a ligand. Last, in terms of cancer therapy, our understanding of the basic mechanisms underlying ErbB-dependent tumorigenesis have led to a major focus on the receptors as targets for therapeutics. Given the perception of multilayered signaling through the ErbB family, this choice appears to be valid, but future targeting of additional components of the ErbB network is expected to increase clinical success in the future.

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